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Manipulation of the Tumour Microenvironment in Chronic Lymphocytic Leukaemia Investigation of a Novel Therapeutic Strategy

Cuthill, Kirsty May

Awarding institution:
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**Manipulation of the Tumour Microenvironment in
Chronic Lymphocytic Leukaemia:
Investigation of a Novel Therapeutic Strategy**

Kirsty Cuthill

A Thesis Presented for the Degree of
Doctor of Philosophy
King's College London
2017

Declaration

I hereby declare that I alone composed this thesis and the work is my own, except where stated otherwise.

Kirsty Cuthill

February 2017

Abstract

The aim of this thesis was to investigate whether manipulation of the tumour microenvironment can suppress the proliferation of malignant cells in Chronic Lymphocytic Leukaemia (CLL). It has been established the CLL cells undergo proliferation in the lymph-node compartment, where they interact with components of the tumour microenvironment including activated CD4 T cells. We therefore explored whether T cell suppression using ciclosporin (CsA) could inhibit the activation and proliferation of CLL cells.

A series of *in-vitro* assays was developed to characterise the effect of CsA on CD4 T cells in CLL. In view of the fact that CsA inhibits NFAT (Nuclear Factor of Activated T cells), which plays a role in B cell receptor (BCR) signalling in CLL, we investigated whether CsA has a direct effect on malignant cells. Having shown that CsA has a major T cell-dependent effect and a smaller direct effect on the activation of CLL cells *in-vitro*, we developed a Phase 2 clinical trial (CyCLLe) to investigate the effect of CsA on the proliferation of CLL cells in patients with early stage, adverse risk disease.

The proliferation of CLL cells was measured using *in-vivo* deuterium labelling, a novel method of assessing drug effect. Despite the promising *in-vitro* findings, CsA did not inhibit *in-vivo* proliferation.

A further study of *in-vivo* labelling was designed to assess intra-patient variation in proliferation rates in CLL. Using 'pulse-labelling' with deuterated glucose, it was possible to investigate the trafficking of CLL cells between the lymph-node and peripheral blood. Furthermore, it was possible to explore the relationship between sIgM expression, proliferation and trafficking of CLL cells. Importantly, these studies found that in patients with mutated *IgV_H* genes, there is an apparently independent sub-population of cells, characterised by low sIgM expression and low BCR internalisation that is quiescent.

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Acknowledgements

I am particularly grateful to my supervisors, Prof Stephen Devereux and Dr Andrea Pepper, for providing me with the opportunity to perform this research project. Their specialist knowledge, experience and encouragement enabled me to develop the skills necessary to complete this investigation.

It was a privilege to be a member of the Lymphoma Biology Group at King's College Hospital where the collaborative mentality lies at the heart of research. I am thankful to Benedetta Apollonio, Emma Hamilton, Ana Levi, Marta Pasikowska, Piers Patten, Will Townsend and Debby Yallop for their support. A special thank you to Eve Coulter for listening, advising and for continuing this work. Thank you also for the technical support and educational opportunities provided by Dr. Linda Barber, Dr. Shahram Kordasti, Dr. Alan Ramsay, Prof. Shaun Thomas and Mr. Thomas Seidl. I am also grateful to Rajani Challiah and Swaibu Mugambwa for offering the support of the tissue bank and to Winston Vetharoy for helping with cell-sorting.

This work would not have been possible without the major collaboration with Prof. Derek Macallan and Dr. Yan Zhang at St. George's Hospital who were involved in the design of the deuterium labelling studies and performed the gas chromatography mass spectrometry.

I am thankful to Prof. Chris Fegan, Prof. Chris Pepper and Prof. Duncan Baird at University Hospital Wales for their ideas, support, contribution to the CyCLLe study and telomere analysis.

I am grateful for the valuable contributions made by members of the Biomedical Research Centre including Pj Chana, Susanne Heck and Rosamond Nuamah.

I acknowledge the support of Bloodwise who generously funded this investigation.

I am forever indebted to the patients who consented to participate in this research project and for their significant commitment, upon which this project was dependent.

I am grateful to my friends and family, with a special mention to my grandfather who helped to fund my studies. Most importantly, I would like to thank my husband, Stephen, for his love and support.

Abbreviations

Abbreviation	Meaning
AID	Activation Induced cytidine Deaminase
AIHA	Autoimmune Haemolytic Anaemia
APC	Allophycocyanin
APRIL	A Proliferation Induced Ligand
ATG	Antithymocyte globulin
ATM	Ataxia Telangiectasia Mutated
B-ALL	B-Acute lymphoblastic Leukaemia
BAFF	B-Cell Activating Factor
<i>BCL</i>	B-Cell lymphoma
BCR	B Cell Receptor
BIRC-3	Baculoviral IAP Repeat Containing 3
BR	Bendamstine Rituximab
BSA	Bovine Serum Albumin
BTK	Bruton's tyrosine kinase
CCR	C-C motif Chemokine Receptor
CD	Cluster of Differentiation
CLL	Chronic Lymphocytic Leukaemia
CM	Complete Medium
CMV	Cytomegalovirus
ConA	Concanavalin
CR	Complete Response
CsA	Ciclosporin
CTC	Common Toxicity Criteria
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CXCR	C-X-C motif Chemokine Receptor
CY	Cyanine
CyCLLe	Ciclosporin A in Chronic Lymphocytic Leukemia
CYP3A	Cytochrome P450 family 3, subfamily A
DAPI	4', 6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DTT	1,4 Dithiothreitol
EBV	Epstein Barr Virus
ECG	Electrocardiogram
ECL	Enhanced chemo-luminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assays
ERK	Extracellular signal- related kinase
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum
FCR	Fludarabine Cyclophosphamide and Rituximab
FCR	Fludarabine Cyclophosphamide Rituximab
FDA	Food and Drug Administration
FISH	Fluorescence <i>in-situ</i> hybridization
FOXP3	Forkhead Box P3
GCMS	Gas Chromatography Mass Spectrometry
GFR	Glomerular Filtration Rate
HEV	High Endothelial Venule
HRP	Horse Radish Peroxidase
HSC	Haematopoietic Stem Cell
HTA	Human Tissue Authority
HTA	Human Tissue Authority
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IgH	Immunoglobulin Heavy Chains
IgL	Immunoglobulin Light Chains
<i>IgV_H</i>	Immunoglobulin Variable Heavy Chain
IKK	Inhibitor of NFκB kinase
IL	Interleukin
IRF	Interferon Regulatory Factor

ITAM	Immunoreceptor tyrosine based activation motif
ITP	Immune Thrombocytopenia
IWCLL	International Workshop on Chronic Lymphocytic Leukaemia
KCL	King's College London
KD	Kilodalton
LDH	Lactate Dehydrogenase
LFA	Lymphocyte Function Associated Antigen
LPS	Lipopolysaccharide
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
MAPKK	MAPK Kinase
MBL	Monoclonal B Lymphocytosis
<i>MCL</i>	Myeloid Cell Leukaemia
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MRD	Minimal Residual Disease
MYD88	Myeloid Differentiation Primary Response 88
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor kappa B
NK	Natural Killer
NLC	Nurse Line Cell
NRES	National Research Ethics Committee
NRES	National Research Ethics Service
NSG	Non-obese diabetes/severe combined immunodeficiency/γc ^{null}
ORR	Overall Response Rate
OS	Overall Survival
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PD	Programmed Death
PDL	Programmed Death Ligand
PE	Phycoerythrin
PERCP	Peridinin Chlorophyll Protein
PFA	Paraformaldehyde
PFS	Progression Free Survival
PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PLC	Phospholipase C
PLC	Phospholipase C
PMA	Phorbol Myristate 13-Acetate
PMT	Photomultiplier Tube
PRCA	Pure Red Cell Aplasia
PRR	Pathogen Recognition Receptor
PTFE	Polytetrafluoroethylene
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
S1P	Sphingosine-1-phosphate
S1P1	Sphingosine-1-phosphate-receptor-1
SDF	Stromal Derived Factor
SDS	Sodium Dodecyl Sulfate
SEER	Surveillance, Epidemiology and End Results Program
SF3B1	Splicing Factor 3b Subunit
STELA	Single telomere length analysis
sIgM	Surface Immunoglobulin M
SLL	Small Lymphocytic Leukaemia
SYK	Spleen Tyrosine Kinase
T _{CM}	Central Memory T-Cell
T _{EM}	Effector Memory T-Cell
T _{EMRA}	Terminal Differentiated Effector Memory T-Cell
TAA	Tumour Associated Antigen
TAP	Trials Acceleration Program

TCR	T cell receptor
Tfh	T-follicular helper
TGF- β	Transforming Growth Factor Beta
Th	T helper
TK	Thymidine Kinase
TLR	Toll-like Receptor
TP53	Tumour Protein 53
Treg	T regulatory
UHW	University Hospital Wales
v/v	Volume/ Volume
V β	Variable Beta
VC	Vehicle Control
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WHO	World Health Organisation
B2-M	Beta-2 microglobulin
7-AAD	7-Aminoactinomycin D

Chapter 1 . Introduction

1.1 The Human Immune System

The immune system is critical in maintaining the integrity of the human body through recognition and elimination of foreign substances and organisms. Robust discrimination between 'self' and non-self' forms the foundation of the immune system in all organisms; from the most basic to the most complex system that protects human beings.

The human immune system has evolved into a highly sophisticated defence system that includes both immediate (innate) and specific (adaptive) components with 'memory' that enables enhanced efficiency following repeated pathogen exposure.

In addition to protecting against infection, the immune system provides an early response system to recognise and eliminate malignant cells before they undergo tumorigenesis. However, it is becoming increasingly recognised that cancers are able to bypass, and in some cases co-opt the immune system to support their survival and proliferation.

1.1.1 Phylogeny

In its simplest form, immunity is evident in marine sponges, where 'grafts' from sponges of a different colony are recognised as non-self and are rejected. The protein structure motif that permits antigen recognition (pathogen recognition receptors) has been highly conserved throughout evolution and is evident in plants, invertebrates and human beings.

Adaptive immunity has evolved in the lower, jawless vertebrates including the lamprey, in which lymphocyte-like cells express a receptor that mediates the immune response. T and B cell responses are well defined in jawed vertebrates in which the development of separate sites of differentiation appears to have contributed to evolution of the immune system. The thymus is evident in teleosts (bony fish, amphibians, reptiles, birds and mammals), where there is evidence of major histocompatibility complex (MHC) molecules, cell-mediated immunity and cytotoxic T cells. At the highest level of evolution of the immune system, warm-blooded

vertebrates have germinal centres that enable T cell dependent, high affinity secondary antibody responses.

1.1.2 Ontogeny

Cells of the human immune system, in addition to red blood cells and megakaryocytes, originate from haematopoietic stem cells (HSCs) that are characterised by the property of self-renewal. During embryogenesis, haematopoiesis takes place in the yolk-sac, followed by the fetal liver and eventually the bone marrow where HSCs receive growth and differentiation signals from the bone marrow stromal cells.

T cell progenitors that lack CD4 and CD8 expression travel to the thymus and under the influence of chemokines, they migrate through the cortex where they express a randomly generated T cell receptor (TCR) and switch on the expression of both CD4 and CD8. The TCR is generated by rearrangement of V-, D- and J- region genes to form the α and β chains that are required for antigen specificity. CD4 and CD8 expression is necessary for the recognition of antigen presented by MHC and determines whether the cells become T helper or cytotoxic T cells respectively. T cells subsequently undergo 'thymic education' that involves both negative and positive selection so that they exit the thymus bearing either CD4 or CD8 and a TCR that can recognise foreign antigen bound to MHC. Negative selection of strongly self-reactive T cells in the thymus underpins immune 'tolerance'.

A smaller population of T cells ($\gamma\delta$ T cells) arises earlier in thymic development; these cells do not require antigen to be presented by MHC and do not express CD4 or CD8; they are thought to play an immune regulatory role.

B lymphocyte precursors (pro-B cells) are dependent upon on the expression of *E2A*, early B cell factor, and the *PAX 5* gene that encodes B cell activator protein for development into pre-B cells. The B-1 subset of progenitors that expresses surface IgM (sIgM) and CD5 moves from the fetal liver to the peritoneal cavity early in neonatal period, where these cells produce low affinity, multi-specific IgM (natural antibodies) in the absence of antigen. Conventional B cells (B-2 cells) express lower levels of surface IgM and lack CD5 expression and represent a separate developmental lineage.

B cells develop their specificity in a specific sequence. The D-J segments of both alleles of the heavy chain Immunoglobulin loci rearrange followed by a single V-DJ rearrangement. This is followed by V-J recombination of first one kappa light chain and then the other. If this is not productive, an attempt is made to achieve a productive lambda light chain allele rearrangement. This is followed by the synthesis of sIgM, which suppresses any further gene rearrangement by exclusion of non-rearranged alleles so that each B cell can only express one heavy chain and one light chain. This forms the basis of clonal selection.

As in T cell mediated tolerance, deletion of auto-reactive B cells and anergy contribute to tolerance in B lymphocytes. Evidence for B cell anergy derives from the double transgenic mice made to express both soluble lysozyme and a high affinity antibody to lysozyme. The mice were tolerant and could not be immunised to produce anti-lysozyme; although the antibody was present on the surface of the B lymphocytes, it was not secreted in the serum. In the case of soluble proteins, T cells are more easily tolerised such that B cells cannot be triggered to produce high affinity antibody as the T cells required for T-B help are already tolerant.

Natural killer cells that provide innate immunity arise from HSCs in the bone marrow and from early lymphoid precursors in the thymus. Maturation into functional NK cells requires the expression of inhibitory receptors that recognise 'self' MHC, following which they migrate to mucosal surfaces where they may encounter antigen.

1.1.3 The Normal Immune Response

The first line of defence against infectious agents in vertebrates is a physical barrier; skin provides the external barrier whilst mucous secretions covering the epidermal layers of the inner surfaces of the respiratory, digestive and reproductive tracts provide the internal barrier.

Secondly, innate immunity provides the immediate but non-specific defence against infectious agents through recognition of the highly conserved pathogen associated molecular patterns (PAMPs). The Toll-like receptors (TLRs), positioned in the plasma membrane of sentinel cells including macrophages and dendritic cells, constitute a major sub-set of the pathogen recognition receptors (PRRs). They recognise PAMPs

and trigger the activation of transcription factors including nuclear factor kappa B (NFκB) and members of the interferon regulated factor (IRF) family, leading to the expression of cytokines and chemokines. Macrophage and neutrophil mediated phagocytosis, first described by Metchnikoff in 1883, involves the engulfment and digestion of microorganisms. The complement system, again reported at the turn of the twentieth century, involving a cascade of proteins contributes to the elimination of microorganisms via direct lysis and opsonisation (coating) that enhances phagocytosis. Additionally, lysozyme, a muramidase enzyme that splits the peptidoglycan bacterial wall, provides an important component of the immediate defence.

The innate immune system triggers the activation of the adaptive immune system, composed of B and T lymphocytes, and provides critical information regarding the nature and duration of response that is required to eliminate the pathogen. Dendritic cells provide the conduit between innate and adaptive systems; first described by Steinman and Cohn in 1973, they continuously sample the environment by pinocytosis and migrate to the lymph-node, where they present antigen via MHC to T lymphocytes. In addition to flagging the presence of a pathogen via antigen presentation, the antigen presenting cells provide a second signal (co-stimulation) via expression of CD80 and CD86 that is necessary for the successful activation of naïve T lymphocytes (Owen, Punt, Stranford 2013).

1.1.4 Lymphocyte Trafficking

Immune surveillance is provided by lymphocytes that continuously traffic between the peripheral blood and lymph-node compartments in search of antigen. Using radioactively labelled lymphocytes that were injected into the circulation of rats, Sir James Gowans demonstrated that lymphocytes rapidly migrate into lymph-nodes by crossing high endothelial venules (HEVs) and recirculate between blood and lymph-node up to two times per day.

Naïve lymphocytes 'home' to lymph-nodes via a multistep adhesion cascade. Primary adhesion (rolling), via interaction between L-selectin expressed by lymphocytes and glycoproteins expressed by HEV cells is transient and unstable but allows the lymphocyte to sample the environment. Naïve T cells express CCR7 and CXCR4 (

receptors for CCL21 and SDF-1 respectively) and naïve B cells express additionally express CXCR5 (receptor for CXCL13), ligation of which leads to chemokine induced activation. Conformation change in LFA-1 (lymphocyte function antigen 1) subsequently leads to stable attachment to ICAM1 and ICAM 2 (intercellular adhesion molecules 1 and 2). Lymphocytes subsequently 'crawl' along the HEV before transmigrating across the endothelium (diapedesis) and undergoing chemotaxis within the cortex.

The lymph, which contains foreign antigens present in tissues, enters the subcapsular sinus via the afferent vessels. Within the lymph-node, B and T lymphocytes are relatively separated into different structures; B lymphocytes are located in follicles that consist of a mantle of resting small B lymphocytes and a pale germinal centre containing mostly proliferating B cells and few T cells. Following exposure to antigen, the primary follicles develop into secondary follicles with highly proliferative germinal centres. Recruitment of lymphocytes to the germinal centre depends on the local expression of SDF-1 that attracts cells expressing the chemokine receptor CXCR4.

Antigen-activated lymphocytes are held within the lymph-node for a period of approximately three days whilst naïve lymphocytes that do not interact with antigen leave via efferent lymphatics. S1P (sphingosine 1 phosphate) and its receptor S1P1 (sphingosine 1 phosphate receptor type 1) have been shown to play a key role in lymphocyte egress. Expression of S1P1 is cyclical; high S1P levels in the lymph and blood leads to S1P1 internalisation and down regulation, whilst within the lymph-node parenchyma, expression is upregulated. Egress from the lymph-node results from an S1P gradient in cortical sinuses that allows lymphocytes to overcome retention mediated by G α i-coupled receptors (CCR7 and CXCR5).

The spleen is divided into the white pulp (secondary lymphoid tissue) and red pulp which is responsible for removal of red cells, platelets and some blood borne pathogens via phagocytosis. Plasmablasts and plasma cells are present in the marginal zones.

1.1.5 Antigen Recognition

The specific acquired immune system depends on the recognition of 'antigen' via antigen-receptors expressed by B and T lymphocytes as well as antibody that is secreted by mature B lymphocytes (plasma cells). The 'antibody formation theory' was first described by Ehrlich in 1900 and was further developed by Marrack who described the antigen-antibody binding hypothesis in 1938. Antibodies not only recognise antigens but they recruit components of the immune system to potentiate the immune response. The structure of antibodies was elucidated by Porter and Endelman in 1959-1962; critically it was shown that antibodies have a 'variable region' involved in antigen recognition and a 'constant region' that engages with complement and macrophages to effect a response. Antibody-coated microorganisms are therefore susceptible to elimination via complement-mediated lysis and by phagocytosis with enhanced affinity.

Each B lymphocyte produces a specific antibody and expresses a trans-membrane version of the antibody on its cell surface to act as a receptor for the specific antigen. Following receptor ligation, the plasma cell secretes antibody that is specific to the antigen. Clonal selection of the B lymphocytes that are triggered by antigen ligation leads to successive rounds of proliferation to provide sufficient quantities of antibody to eliminate the infection. Subsequent exposure to the microorganism stimulates a faster response of greater magnitude that demonstrates immunological 'memory'. Indeed, this is the basis of vaccination, first described by Jenner in 1796 in the context of smallpox and later developed in 1878 by Pasteur, who developed a live-attenuated vaccine against rabies.

Inadvertent recognition of 'self' antigens is pathological and is the basis of auto-immune disease. In health, this is avoided by 'tolerance', a permanent state of unresponsiveness to self that involves the suppression of self-reacting lymphocytes by a number of mechanisms including thymic deletion and the adoption of an 'anergic' state.

B lymphocytes rely on help from activated CD4⁺ T cells to generate high affinity antibody and to under-go class switching; in the absence of the thymus, IgM responses

are low affinity and exhibit limited memory. B lymphocytes become activated following antigen engagement of the B cell receptor (BCR). Propagation of the activation signal requires a cascade of signalling that is initiated upon the recruitment of the BCR to the lipid raft; this allows LYN to phosphorylate the ITAM domains of the cytoplasmic tails of the BCR-associated immunoglobulin, which leads to the recruitment and activation of SYK and BTK. In turn, this activates the transcription factors NFAT and NF κ B that leads to the expression of interleukins and interferons and to proliferation and differentiation. Efficient BCR effector-responses are enhanced by the B cell co-receptor complex, comprised of CD19, CD21, CD81 and CD225, that reduces the threshold for activation of the BCR. Secondly, T cell dependent B cells receive co-stimulation from T-helper cells that express CD40L following antigen presentation by B cells with MHC class II. CD40L engagement with CD40 expressed by B cells enables full activation, somatic hypermutation and immunoglobulin class switching.

T lymphocytes provide cell-mediated immunity, providing defence against intracellular pathogens. Antigen recognition via the T cell receptor (TCR), only recently discovered in 1983, requires the presentation of processed antigen with MHC. Cytotoxic T cells recognise peptides presented by MHC class I, present on most nucleated cells, and T-helper and regulatory cells recognise MHC class II, present on 'professional' antigen presenting cells including dendritic cells, macrophages and B lymphocytes. Naïve T cells only recognise antigen presented by dendritic cells, whereas primed T cells can recognise antigen presented by macrophages and B lymphocytes.

In addition to TCR-mediated T cell activation, a second co-stimulatory signal (CD28) is required to fully activate resting T-helper cells and to induce the cells to enter the cell cycle. The B7 family ligands (CD80 and CD86) expressed by mature dendritic cells provide potent co-stimulation via binding with the CD28 receptor.

Following receptor ligation, CD4 or CD8 is recruited into the CD3/ CD3 co-receptor complex and a signalling cascade is activated that involves the RAS/ MAP kinase and phosphatidylinositol signalling pathways.

Antigen-driven T cell activation and proliferation gives rise to clonal expansion and differentiation to cytotoxic T-effectors, T-helpers and regulatory T cells (Tregs) as well as producing an enlarged population of memory cells. T helpers become polarised towards a response to intracellular pathogens (Th1), a response to extracellular antigens (Th2) or an acute inflammatory response against extracellular bacterial and fungal infections (Th17), each characterised by a specific cytokine profile.

Following activation, a number of mechanisms operate to dampen T-cell activity that act at the level of the T cell itself or involve other T-subsets (Tregs). Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is expressed following the activation of T cells and acts as a repressor of activation by engaging CD80 and CD86 with higher affinity than the co-stimulatory molecule CD28. In a similar fashion, PD-1 expressed by chronically activated T cells engages PDL-1 and PDL-2 expressed by antigen presenting cells and inhibits further T-cell activation and IL-2 production. Notably, this axis has been co-opted by cancer cells e.g. melanoma and Hodgkin's lymphoma cells that evade immune recognition by silencing T-cells.

Following successful eradication of the antigen, most of the B and T lymphocytes die by apoptosis; however a small proportion with the highest antigen affinity survival providing 'immune memory'.

1.1.6 Diseases of the Immune System

The human immune system has evolved to detect and to provide a tailored response to wide range of pathogens and tumour antigens. Disease arises from failure of the immune system to respond appropriately; this may be due to dysfunction (either inherited or acquired) or due to the development of evasive strategies by pathogens and tumours. Conversely, disease can arise from over-activation of the immune system, which can lead to hypersensitivity reactions and to haematological malignancies. Failure of immunological tolerance mechanisms gives rise to autoimmune disease.

1.1.7 Immunosenescence

The immune system undergoes significant change over time, a process that has been termed immunosenescence and contributes to an increased risk of infection, malignancy, autoimmunity and an impaired response to vaccination with age. Changes are most noticeable in the T cell component of the adaptive immune system, where there is a proportionate increase in memory T cells in both CD4 and CD8 compartments and a reduction in naïve T cells due to involution of the thymus with age and ongoing exposure to antigen.

Longitudinal studies have demonstrated that an inverted CD4/ CD8 ratio associated with expansion of CD8+ CD28- T cells, reduced proliferative response to T cell mitogens, low B cell counts and cytomegalovirus (CMV) seropositivity is predictive of mortality (Olsson et al., 2000, Wikby et al., 2002). However, it is likely that pathogens other than CMV play a role in the chronic antigen stimulation that contributes to the age-related changes.

1.1.8 Tumour Immunology

Tumours arise from a multistep process that overcomes the mechanisms that normally protect cells from mutagenesis and unchecked proliferation, culminating in a growth advantage. Evasion of the immune system via the expansion of non-immunogenic clones is now recognized as a hallmark of cancer.

Three phases of tumour evolution have been described, involving 'elimination', 'equilibrium' and 'escape' phases whereby the tumour cells progressively acquire mechanisms to evade the immune system over time (Pizzi, Boi, Bertoni, & Inghirami, 2016). The initial phase of elimination involves the recognition of tumour associated antigens (TAAs) and stress molecules and elimination by NK cells and cytotoxic T cells. The second phase of equilibrium allows sub-populations of non-immunogenic cells to emerge and survive whilst those expressing TAAs are eliminated. Eventually the tumour escapes immune recognition by loss of TAAs, inhibition of antigen processing and expression of MHC, the development of an immunosuppressive microenvironment, up-regulation of regulatory T cells and suppression of T-effector activity. The profound efficacy of immune-checkpoint inhibitors in solid tumours and

some haematological malignancies has recently highlighted the importance of immune-evasion in tumour biology.

1.1.9 Lymphoid Neoplasia

It is now clear that many, if not the majority of lymphoid neoplasms arise from dysfunctional lymphocyte development or from persistent or abnormal immune responses. B cell neoplasms mimic stages of normal B cell differentiation; precursor B cell neoplasms e.g. B-acute lymphoblastic leukaemia (B-ALL) arise from aberrant immunoglobulin gene rearrangement. Mantle cell lymphoma is thought to correspond to CD5+ naïve B cells whilst germinal-centre derived lymphomas e.g. follicular lymphoma arise during somatic hypermutation and class-switch recombination. Finally, post germinal-centre type lymphomas arise from ongoing BCR activation due to mutations as in activated B cell type diffuse large B cell lymphoma, or antigen/autoimmunity as in marginal zone lymphoma.

As in the normal immune system, lymphoid tumours are subject to regulatory influences from other cells in the microenvironment including macrophages, endothelial cells, stromal cells and T-cells.

In this thesis, I will focus on chronic lymphocytic leukaemia, a common neoplasm of mature B lymphocytes in which both BCR signalling and microenvironmental influences, particularly T cells play a major role.

1.2 Chronic Lymphocytic Leukaemia

1.2.1 Epidemiology

Chronic lymphocytic leukaemia (CLL) is the most prevalent leukaemia in the Western Hemisphere. It is a disease of the elderly with an incidence of 0.1 cases per 100,000 rising to 32 cases per 100,000 between the ages of 30 and 80 years (Howlader, 2014). Men are more commonly affected and the disease is rare in people of Asian origin. In view of the aging population and improved survival following diagnosis, CLL is of increasing socioeconomic importance.

1.2.2 Cell of Origin

CLL cells are characterised by the co-expression of CD19, a B cell lineage marker, and CD5, a T cell lineage marker, and the cell of origin is a subject of ongoing investigation. Recent global gene expression studies have compared CLL cells with peripheral blood naïve B cells, splenic marginal zone B cells, mature CD5+ B cells, class-switched and IgM+ memory B cells. The investigators found that CLL cells are most similar to mature CD5+ B cells, a minor B cell subset in healthy individuals (Macallan et al., 2009; Seifert et al., 2012).

In 60-65 % cases of CLL, the immunoglobulin heavy chain variable region genes (*IgV_H*) are mutated, reflecting that T cell dependent somatic hypermutation has taken place in the lymph-node germinal centre. In the remainder of cases, *IgV_H* is unmutated, reflecting germinal centre-independent maturation. Gene expression studies investigating the cell of origin, demonstrated that CLL cells with mutated *IgV_H* were most similar to a subset of CD5+ B cells that expressed CD27 and have *BCL6* mutations, demonstrating that they have undergone a germinal centre reaction. CLL with unmutated *IgV_H* genes are most similar to mature CD5+ B cells that are CD27- and have not undergone a germinal centre reaction (Borel, Feurer, Gubler, & Stähelin, 1976; Seifert et al., 2012).

A major contribution to our current understanding of the pathogenesis of CLL was the finding that in one third of cases, there is highly restricted *IgV_H* gene use by the B cell receptor (BCR) (Agathangelidis et al., 2012). Restricted *IgV_H* gene usage provides strong

evidence that in some cases, the tumour is driven by distinct antigens, hypothesised to include viral and bacterial antigens and auto-antigens expressed by dying cells (Chu et al., 2008; Lanemo Myhrinder et al., 2008; Steininger et al., 2012). A number of distinct stereotypes have been described with quasi-identical variable heavy chain complementarity determining region 3 (H-CDR3) sequences and common biological and clinical characteristics (Agathangelidis et al., 2012).

In addition to the widely accepted theory that chronic stimulation of B cells by extrinsic antigen drives CLL pathogenesis, a recent study has identified that CLL BCRs are capable of antigen-independent cell autonomous signalling (Minden et al., 2012). Transplantation of CLL derived H-CDR3 (an internal epitope of the BCR) into BCR-deficient cells induced calcium signaling, which was abolished by the introduction of mutations into the H-CDR3. It is possible that both antigen-dependent and antigen-independent signalling cascades are active in CLL pathogenesis.

1.2.3 The Genetic Landscape of CLL

In CLL, it has been documented that there is a comparatively small number of genetic mutations within the malignant clone. Whole exome sequencing (WES) and whole genome sequencing (WGS) on large cohorts of patients with CLL have recently identified recurrent mutations (Wang et al., 2011), in turn providing important insight into disease pathogenesis. The most frequent mutations and their proposed role in the biology of disease are listed in Table 1.1. These mutations can be considered as founder mutations, i.e. present from the outset, or as secondary driver mutations, occurring as a later event.

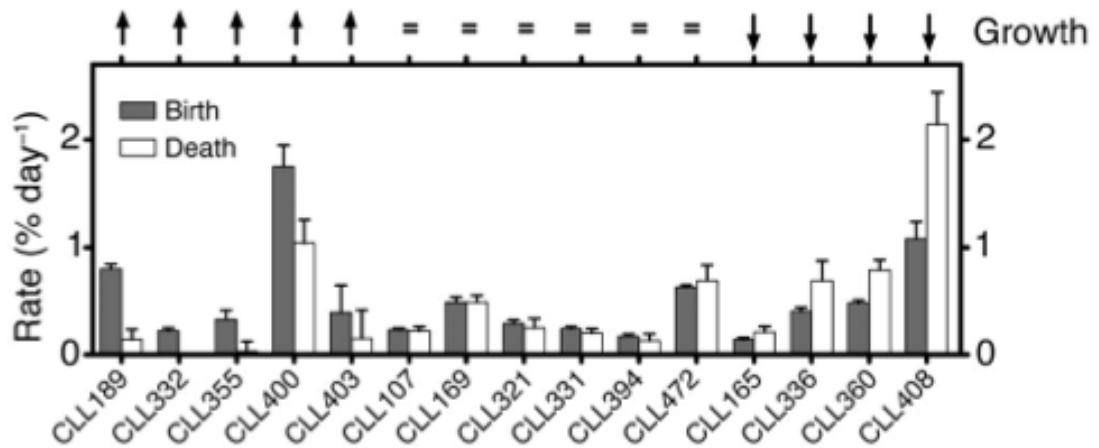
Longitudinal studies that repeated WES after a median of 3.5 years have demonstrated that in a proportion of patients, sub-clonal evolution takes place where there is an expansion of sub-clones towards clonality. Although this may be accelerated by chemotherapy which is thought to allow the expansion of aggressive subclones following removal of the incumbent clone, this phenomenon may also take place in the absence of therapy (Landau et al., 2013). The presence of subclonal driver mutations has been demonstrated to be an independent risk factor for rapid disease progression.

Table 1.1 Frequently Occurring mutations in CLL

Mutation	Frequency	Association	Pathway	Reference
ATM	10-15 %		DNA damage repair	(Guarini et al., 2012)
NOTCH-1	5-11 %	Trisomy 12	NOTCH	(Sportoletti et al., 2010) (Puente et al., 2011)
MYD88	3 %			(Puente et al., 2011)
SF3B1	15 %	11q del	Spliceosome	(Wang et al., 2011) (Quesada et al., 2011)
BIRC-3	4 %		NFκB	(Rossi et al., 2012)

1.2.4 Pathogenesis

For many years, CLL was considered to be a disease of failed apoptosis, characterised by accumulation of cells arrested in G0-G1 stage of the cell cycle. Up-regulated expression of the anti-apoptotic genes *BCL2* and *MCL1* supports this theory (Hanada, Delia, Aiello, Stadtmauer, & Reed, 1993; Johnston et al., 2004). However, in 2005, *in-vivo* labelling studies using deuterated water in 19 patients with CLL revealed substantial proliferation rates of up to 2 % of the tumour clone each day, as illustrated in Figure 1.1 (Messmer et al., 2005). Patients with progressive disease generally had proliferation rates of ≥ 0.35 % per day. Lin et al went on to report that CLL telomere length was the shortest ever recorded in primary tissue and that extensive erosion was associated with telomere dysfunction, genetic instability and disease progression (Lin et al., 2009). Furthermore, proliferation is a necessary factor for the accumulation of mutations that provide a selective advantage leading to evolution of the CLL genome.



Messmer et al 2005

Figure 1.1 CLL birth and death rates measured using *in-vivo* deuterium

Recognition that proliferation is a key feature in CLL pathogenesis prompted investigators to further define the factors that drive proliferation of CLL cells. A major contribution to this work was the finding that proliferation takes place in the CLL lymph-node. Gene expression studies that compared the lymph-node, bone marrow and peripheral blood compartments identified that there was upregulated expression of genes associated with BCR signalling in the lymph-node compared to the peripheral blood and that gene expression was greatest in cases of CLL with unmutated *IgV_H* genes (Herishanu et al., 2011). Consistent with this finding, CLL proliferation measured by Ki67 expression was four fold greater in the lymph-node than in the peripheral blood and there was a trend towards increased Ki67 expression in unmutated CLL lymph-nodes (Herishanu et al., 2011).

1.2.5 The CLL Microenvironment

The survival and proliferation of CLL cells is dependent on complex interactions with components of the tumour microenvironment. This is clearly evidenced by the fact that CLL cells cultured *ex-vivo* rapidly undergo apoptosis but can be rescued by co-culture with stromal cells or endothelial cells, a phenomenon that is not observed with healthy B lymphocytes (Buggins et al., 2010; Lagneaux, Delforge, Bron, De Bruyn, & Stryckmans, 1998; Panayiotidis, Jones, Ganeshaguru, Foroni, & Hoffbrand, 1996). Activated CD4⁺ T cells have also been demonstrated to promote CLL cell survival, activation and proliferation (Ghia et al., 2005; Patten et al., 2005; Ranheim & Kipps, 1993). The pathological features of T cells in CLL will be further explored in section 1.2.8.

1.2.6 B Cell Receptor Signalling in CLL

The role of B cell receptor signalling in the pathogenesis of CLL has been a major area of focus since it became apparent that the mutational status of *IgV_H* genes was recognised to be an important determinant of disease course (Damle et al., 1999; Hamblin, Davis, Gardiner, Oscier, & Stevenson, 1999; Stevenson, Krysov, Davies, Steele, & Packham, 2011).

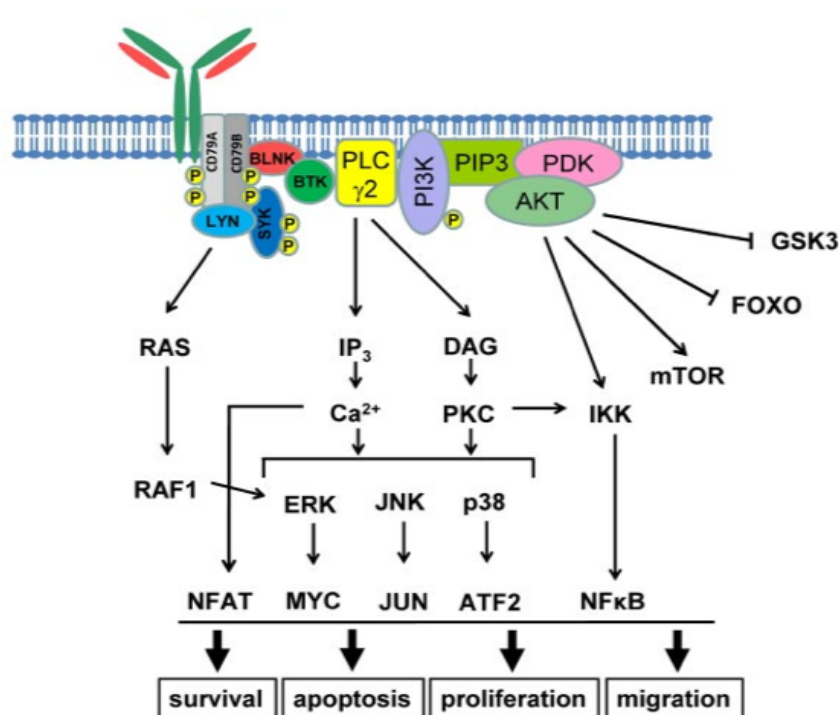
The BCR is composed of antigen-binding heavy chains (IgH) and light chains (IgL) that are non-covalently coupled to Ig α (CD79a) and Ig β (CD79b) subunits. Upon antigen-engagement, there is clustering of BCRs that leads to tyrosine phosphorylation of immunoreceptor tyrosine based activation motifs (ITAMs) in the cytoplasmic tails of CD79a/b by src family kinases. This leads to recruitment of SYK that activates a signalling cascade that engages BTK, PI3 kinases, NF κ B, PLC γ , NFAT, MAP kinases and RAS (see Figure 1.2). This signalling culminates in the survival and proliferation of the malignant cell.

The ability of a CLL cell to signal through the BCR depends not only on the strength of the signal but on the disease biology i.e. the *IgV_H* mutational status (Mockridge et al., 2007). CLL cells with unmutated *IgV_H* genes are generally more responsive to stimulation through the BCR, with the strength of the BCR signal partially reflecting the degree of sIgM expression (Mockridge et al., 2007). CLL cells with mutated *IgV_H* genes

have been characterised to have poor BCR mediated signalling capacity, with low sIgM expression, inability to mobilise calcium upon antigen engagement and by constitutive activation of ERK and NFAT (Apollonio et al., 2013). In healthy B cells, anergy functions to silence autoreactive B cells upon low affinity binding with self-antigen, culminating in these cells undergoing apoptosis (Goodnow, 1997). However, CLL cells express anti-apoptotic proteins, including BCL2 and MCL1, leading to prolonged survival despite an anergic phenotype. The state of anergy in CLL can be overcome by inhibition of NFAT signalling using the cell-permeable VIVIT peptide that prevents nuclear translocation of NFAT. Following reversal of anergy, CLL cells rapidly undergo apoptosis. Furthermore, NFAT inhibition using the VIVIT peptide in the MEC-1 transplanted xenograft *Rag2*^{-/-} γ *c*^{-/-} mouse model has been shown to delay tumour growth and prolong survival (Apollonio et al., 2013).

A direct relationship between signalling from the tumour microenvironment and BCR expression and downstream signalling by CLL cells has recently been proposed whereby interleukin 4 (IL4), expressed by T cells, restores CD79b expression, sIgM expression and BCR signalling and that this finding is most pronounced in cases with unmutated *IgV_H* genes (Aguilar-Hernandez et al., 2016; Guo, Zhang, Chiorazzi, & Rothstein, 2016).

The overwhelming clinical success of drugs that inhibit BCR signalling has emphasised the importance of the BCR in CLL pathogenesis. However, it must not be overlooked that in addition to inhibiting BCR induced proliferation, BTK inhibition with ibrutinib and PI3K inhibition with idelalisib additionally abrogates the protective effect of the tumour microenvironment (Herman et al., 2011; 2010).



(Stevenson et al., 2011)

Figure 1.2 Principal downstream signalling pathways linking the BCR to biological pathways in CLL

1.2.7 Trafficking of CLL cells

It is currently believed that CLL is a two-compartment disease. In the lymphnode compartment, interaction with the microenvironment and signalling through the BCR enables CLL cells to proliferate, after which they are released into the peripheral blood compartment where they receive survival signalling from the vascular endothelium (Buggins et al., 2010). It is thought that some cells re-circulate to the lymph-node but evidence for this is lacking. Characterisation of trafficking is essential to fully understand the pathophysiology of CLL, particularly as disruption of trafficking is thought to contribute to the efficacy of the novel agents in CLL. Furthermore, it is necessary to understand whether CLL cells behave as a uniform clone or whether there is sub-clonal heterogeneity in proliferation and trafficking.

Previous studies have shown that recently proliferated cells express low levels CXCR4, the receptor for the chemokine SDF-1, and high levels of CD5, a marker of recent BCR activation (Calissano & Damle, 2011) CXCR4 has long been regarded as a key molecule

for lymphocyte homing, enabling CLL cells to bind to SDF-1 to enter the lymph-node and bone marrow compartments (J. A. Burger, Burger, & Kipps, 1999). Comparative studies of CXCR4 expression have revealed that expression is lower in the lymph-node than in the peripheral blood (Pasikowska et al., 2016), where it is thought that sIgM internalisation and IL4 signalling downregulates expression (Quiroga et al., 2009; Vlad et al., 2009) (Aguilar-Hernandez et al., 2016). Following release into the peripheral blood, it is hypothesised that CXCR4 is re-expressed, enabling CLL cells to home to the lymph-node. Evidence for this is based on *in-vitro* studies that have shown that CXCR4 is rapidly re-expressed both in CLL cell culture and in the circulation model (Coelho et al., 2013; Walsby et al., 2014), however, *in-vivo* evidence is lacking. Of particular interest is whether CLL cells have an equal capacity to home to the lymph-node to proliferate or whether this function is specific to a proliferative sub-population.

BCR expression has been linked with CXCR4 expression, with the hypothesis that expression is trans-regulated (Coelho et al., 2013). If this is the case, one would expect BCR expression to be lowest in the lymph-node compartment, where antigen engagement induces receptor internalisation. Recently proliferated CLL cells would be characterised by low BCR expression, with subsequent up-regulation in the peripheral blood compartment. However, at odds with this theory is the finding that BCR expression is upregulated in the lymph-node compartment (Coulter et al, manuscript submitted) and that the recently proliferated CXCR4^{lo}/CD5^{hi} sub-population expresses higher sIgM than the resting fraction (Calissano & Damle, 2011). One explanation for this observation is that in the lymph-node, CLL cell expression of CD79b and sIgM is rescued by IL4 signalling from T cells (Aguilar-Hernandez et al., 2016; Guo et al., 2016). Dynamic studies of BCR expression have not been performed *in-vivo*; such studies would provide critical information regarding the kinetics of BCR expression in the lymph-node and peripheral blood compartments. A comprehensive understanding of the dynamics of BCR expression and signalling is particularly relevant in an era when BCR targeting drugs will be increasingly used in clinical practice.

1.2.8 Characterisation of T Cells in CLL

It is evident that although the absolute number of T cells is increased in untreated patients, there is a reduction in the proportion of naïve CD4 and CD8 T cells. Furthermore, in early-stage patients with progressive disease, there is an inversion of the normal CD4:CD8 T cell ratio, with expansion of the CD8+ compartment and skewing towards an effector-memory PD-1 positive phenotype, consistent with a state of replicative senescence (Nunes et al., 2012).

Comparative gene expression profiling of peripheral blood derived CLL T cells with healthy T cells demonstrated that key differences in gene expression in CD4 T cells involved skewing of T cell differentiation towards Th2 phenotype. CLL CD8 T cells had impaired cytoskeletal formation, vesicle trafficking and cytotoxicity compared with healthy CD8 T cells (Görgün, Holderried, Zahrieh, Neuberg, & Gribben, 2005). Functionally, these differences in gene expression have been shown to lead to impaired F-actin polymerization and immune-synapse formation (Ramsay et al., 2008). It has been shown that the changes seen in CLL T cells result from direct contact with peripheral blood derived CLL cells and can be induced in healthy T cells (Görgün et al., 2005; Ramsay et al., 2008).

Molecular characterisation of defective interaction between peripheral blood derived CLL cells and T cells has identified that the PD-1: PDL-1 axis plays a role in immune evasion (Ramsay, Clear, Fatah, & Gribben, 2012). PD-1 expression is enhanced in CLL peripheral blood CD4 and CD8 T cells compared with age matched controls and is even higher in the CLL lymph-node where it is thought that engagement with PDL-1 on CLL cells suppresses T cell activation (Brusa et al., 2013)(Yallop et al, manuscript in preparation).

To investigate the process driving PD-1 up-regulation in CLL CD4 T cells, Yallop et al performed T cell receptor V β spectratyping on PD-1 high and PD-1 low subsets of lymph-node and peripheral blood derived CD4+ T cells and demonstrated a loss of TCR diversity in the PD-1 high subset of both compartments. These findings were further corroborated by sequencing of the TCR V β CDR3 regions of CD4+ T cells taken from paired lymph-nodes and the peripheral blood of individual patients. There was a high

degree of commonality between the TCR sequence from the lymph-nodes and divergence from that seen in the peripheral blood. These findings point towards antigen driven activation and proliferation of CD4⁺ T cells within the lymph-node and a subsequent state of replicative senescence.

The role of T cells in CLL pathogenesis has been suggested by the observation that within the CLL microenvironment, recently proliferated CLL cells are closely associated with CD4⁺, CD25⁺ FOXP3⁻ T cells (Patten, Buggins, Richards, Wotherspoon, Salisbury, Mufti, Hamblin, & Devereux, 2008a) . *In-vitro* studies revealed that co-culture of CLL cells with activated autologous CD4 T cells leads to up-regulation of CD38 expression and proliferation of CLL cells (Patten, Buggins, Richards, Wotherspoon, Salisbury, Mufti, Hamblin, & Devereux, 2008a).

Furthermore, it has been found that activated autologous CD4⁺ T cells are essential for CLL cells to successfully engraft, survive, and proliferate in the non-obese diabetes/severe combined immunodeficiency/ γ c^{null} (NSG) mouse model (Bagnara et al., 2011). Autologous CD4⁺ T cells, activated *in-vivo* by alloantigen were not only necessary for the engraftment of CLL cells, they were also necessary for the characteristic behavior of aggressive CLL cells *in-vivo* i.e. CD38 expression and growth in secondary lymphoid tissue.

The mechanism of action CD4 T cells in supporting the survival and proliferation of CLL cells is multifactorial and, in addition to direct contact, involves the secretion of cytokines. First and foremost, it has been found that ligation of CD40 *in-vitro* leads to NF κ B activation and confers a survival phenotype in CLL cells that can be blocked using an anti-CD40L monoclonal antibody (Furman, Asgary, Mascarenhas, Liou, & Schattner, 2000). CD40 ligation also leads to the upregulation of CD38 expression by CLL cells cultured in *in-vitro* (Willimott, Baou, Huf, Deaglio, & Wagner, 2007). *In-vivo*, CD40L+ CD4⁺ T cells have been shown to be present in the CLL lymph-node, where they cluster around Ki67⁺ proliferating CLL cells (Ghia et al., 2002). Lymph-node derived CLL cells activated by CD40L have been found to attract CD40L positive CD4 T cells through secretion of the chemokine CCR22 (Ghia et al., 2002). Furthermore, CLL cells secrete IL6 that leads to skewing of CD4 T cells from Th1 to Th2 phenotype, and stimulates the production of the pro-survival cytokine IL4 (Buggins et al., 2008). Ahearne et al went

on to demonstrate that CD40L + IL4 leads to enhanced proliferation of CLL cells in response to IL21 *in-vitro* (Ahearne et al., 2013). Pascutti et al have subsequently shown that CD40L + IL21 induces a CLL phenotype and can stimulate proliferation of CLL cells in a comparable manner to activated T cells (Pascutti et al., 2013). They found that lymph-node derived CLL cells showed evidence of IL21 stimulation and that there is detectable IL21 expression in the lymph-nodes of some patients with CLL, greatest in CXCR5+, CD40L+ CD4 T cells. Pascutti et al propose that T follicular helper (Tfh) cells, characterised by IL21 secretion, play a role in CLL pathogenesis. This proposition is supported by the observation that Tfh cells, characterised by CD4+CD45RO+CXCR5+ are increased in the peripheral blood of patients with CLL (Ahearne et al., 2013).

In summary, it is clear that interactions between CLL and T cells are complex and bidirectional and are subject to microenvironmental influence i.e. they differ between lymph-node and peripheral blood compartments. In the lymph-node, the site of tumour proliferation, evidence is weighted towards a pro-tumorigenic role of T cells that involves both direct contact and cytokine signalling. Inhibition of T cell activation as a potential therapeutic strategy is therefore an important line of investigation.

1.3 Clinical Aspects of Chronic Lymphocytic Leukaemia

1.3.1 Diagnosis

CLL is a neoplasm of mature B lymphocytes. A clinical diagnosis is made following the detection of $\geq 5 \times 10^9/L$ clonal B lymphocytes that is present for at least three months (Hallek et al., 2008). CLL is accurately distinguished from other causes of a peripheral blood lymphocytosis using a scoring system with one point awarded for each of the following: CD5+, CD23+, FMC7-, CD79b-/weak, sIg weak. A score of at least three points confirms the diagnosis with 97 % accuracy (Moreau et al., 1997). Monoclonal B lymphocytosis, characterised by the presence of $3.5-5 \times 10^9/L$ monoclonal B lymphocytes, is considered to be a precursor to CLL; with 1 % cases progressing to CLL that requires treatment each year (Rawstron et al., 2008).

CLL runs a highly variable course that is best considered as a spectrum of disease. At one end of the spectrum, a patient may be diagnosed with CLL as an incidental finding following a routine blood test and they may remain asymptomatic with a stable lymphocyte count over decades. At the opposite end of the spectrum, a patient may rapidly become symptomatic of progressive disease that requires early treatment. The tempo of disease may change over time, such that a patient with slowly progressive disease may undergo a period of rapid deterioration. It is estimated that 5 % of patients with CLL will undergo high-grade transformation (Richter's Transformation) into a high-grade B cell lymphoma, pro-lymphocytic leukaemia, Hodgkin's lymphoma or acute leukaemia (Tsimberidou & Keating, 2005).

1.3.2 Clinical Features

Clinical features arise as a result of the infiltration of lymph-nodes, liver, spleen and bone marrow with CLL cells. Infiltration leads to painless lymphadenopathy and hepatosplenomegaly. Normal haematopoiesis is impaired as a result of bone marrow infiltration, leading to anaemia, thrombocytopenia and neutropenia. Patients with CLL have dysfunctional immunity that manifests with an increased frequency of infections, an increased risk of malignancy and an increased incidence of auto-immunity (particularly autoimmune cytopenias). Constitutional symptoms of severe fatigue, anorexia, weight-loss and night sweats may be associated with progressive disease.

1.3.3 Prognosis

The clinical outcome of an individual with newly diagnosed CLL depends upon patient-specific factors i.e. age, performance status and presence of co-morbidities, and disease-specific factors. Given the considerable heterogeneity of the disease, it is useful to apply prognostic tests that reliably predict time to first treatment and overall survival. For many years, CLL prognosis has been predicted according to the stage of the disease in combination with the rate of progression i.e. the lymphocyte doubling time. Disease stage is a 'snap-shot' of disease-burden at any one time and is defined according to the presence of lymphadenopathy, hepatosplenomegaly and cytopenias using either the Binet (Binet, Auquier, Dighiero, & Chastang, 1981) or Rai (Rai et al., 1975) Classification Systems as illustrated in Table 1.2. However, over recent years, a multitude of prognostic biomarkers and genetic variables have been identified that more accurately reflect disease biology.

Table 1.2 Prognostic scoring systems in CLL

Rai Stage	Clinical Features	Median Overall Survival	Binet Stage	Clinical Features	Median Overall Survival
0	Lymphocytosis	>150 months	A	≤ 2 lymph node areas	>150 months
I	Lymphocytosis and lymphadenopathy	101 months	B	≥ 3 lymph node areas	84 months
II	Lymphocytosis and hepatosplenomegaly	71 months	C	Anaemia (Hb < 10g/dl) or thrombocytopenia (Plts<100 x 10 ⁹ /dl)	24 months
III	Lymphocytosis and anaemia	19 months			
IV	Lymphocytosis and thrombocytopenia	19 months			

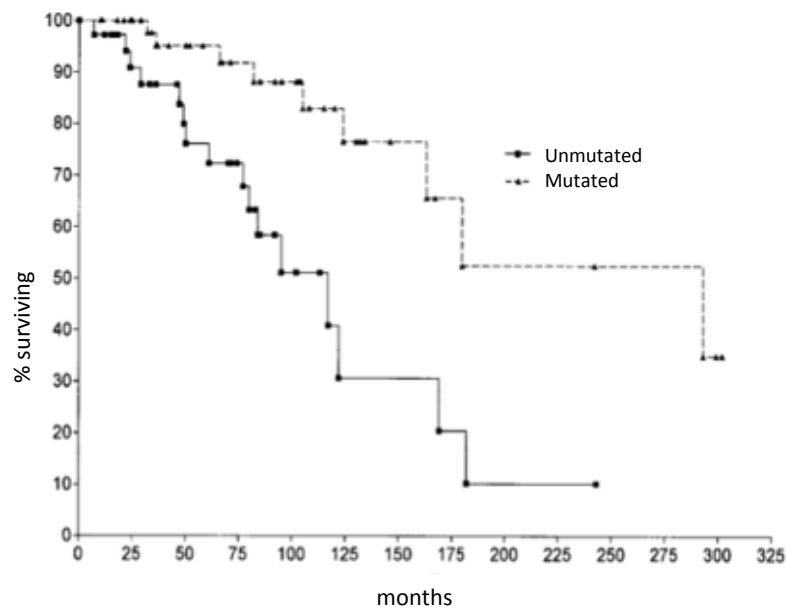
Biochemical Markers

Serum beta 2-microglobulin ($s\beta_2\text{-M}$) is a polypeptide that stabilizes the tertiary structure of the major histocompatibility complex -class 1 (MHC-class 1) expressed on nucleated cells and is physiologically present in extra-cellular fluid. Serum $\beta_2\text{-M}$ levels reflect disease bulk and are predictive of progression free survival (Hallek et al., 1996). As $s\beta_2\text{-M}$ levels are considerably affected by renal function, the prognostic value of this biomarker is increased by adjusting for glomerular filtration rate (GFR) (Delgado et al., 2009).

Serum tyrosine kinase is a cellular enzyme present in proliferating cells and levels have been found to correlate with disease activity and to predict progression free survival. However, tyrosine kinase levels are not routinely measured as conventional assays involve the use of a radioactive isotope.

Immunoglobulin Variable Heavy Chain Gene Mutational Status

The mutational-status of the B cell receptor immunoglobulin variable heavy chain genes (IgV_H) indicates whether the cell has undergone somatic hypermutation in the lymph-node germinal centre. In 1999, Hamblin and Damle independently reported that in approximately one third of patients, the IgV_H genes are unmutated, indicating that the tumour is derived from a naïve B cell. Patients with Binet Stage A disease and unmutated IgV_H genes were found to undergo a more aggressive disease course with a median overall survival of 8 years, compared with a median overall survival of 25 years for patients with Binet Stage A disease and mutated IgV_H genes, as depicted in Figure 1.3 (Damle et al., 1999; Hamblin et al., 1999). Patients with mutated IgV_H genes that use the IgV_H 3-21 genes are an exception, with an unfavourable prognosis, similar to that afforded by unmutated IgV_H genes.



(Hamblin et al., 1999)

Figure 1.3 Kaplan Meier survival curve of CLL patients with Binet Stage A CLL according to IgV_H mutational status

Median survival = 117 months with unmutated IgV_H genes versus 293 months with mutated IgV_H genes ($p=0.001$).

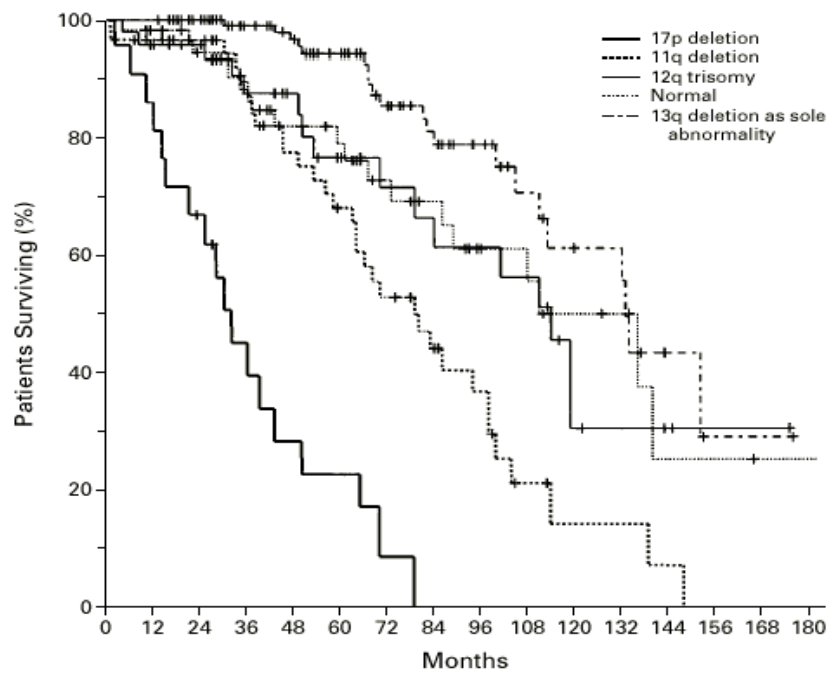
CLL Immunophenotype

Expression of CD38, a type 2 trans-membrane glycoprotein, was reported by Damle *et al* to inversely correlate with IgV_H mutational status with >30 % expression conferring an adverse prognosis (Damle et al., 1999). However, Hamblin et al found that although there is significant correlation between IgV_H mutational status and CD38 expression, there was a discordance in 28 % cases (Hamblin et al., 2002). It has since been established that a threshold of 7 % CD38 expression is a more stringent discriminator of prognosis; with median overall survival estimated to be 114 months in patients with <7 % CD38 compared to 79 months in those with >7 % CD38 expression (Kröber et al., 2002; Thornton et al., 2004).

A recent world-wide multi-centre analysis demonstrated that CD49d, a CLL surface antigen associated with proliferation is the most predictive flow-cytometry based prognostic-marker. CD49d expression of >30 % was associated with a 10 year overall survival of 62 % versus 84 % for those not expressing CD49d (Bulian et al., 2014).

Genetic Factors

Genomic aberrations can be detected in approximately 80 % cases of CLL using Fluorescent *In Situ* Hybridisation (FISH) and have a variable prognostic significance, as illustrated in Figure 1.4. The commonest genomic aberration, affecting 50-55 % cases is 13q deletion. It is considered as an initiating lesion that has the potential to disrupt tumour suppressor function. Monoallelic loss of 13q has a favourable clinical outcome (Dohner et al., 2000), whereas prognosis conferred by biallelic loss remains controversial (Dewald et al., 2003; Garg et al., 2012; Puiggros et al., 2013). The second most common lesion is trisomy 12 (10-20 % newly diagnosed cases), which is associated with an intermediate prognosis. Twenty percent of patients with progressive disease that requires treatment have an 11q deletion, leading to loss of the *ATM* (Ataxia Telangiectasia) tumour suppressor gene in almost all cases. CLL harbouring 11q deletion is characterised by rapid progression, short survival and bulky lymphadenopathy (Dohner et al., 1997). Deletion of the short arm of chromosome 17 affects 3-8 % of patients at diagnosis and 31 % at relapse. In all cases of 17p deletion, there is loss of the *TP53* tumour suppressor gene. In the majority of cases of monoallelic loss of *TP53*, there is a *TP53* mutation in the remaining copy, further contributing to loss of tumour suppressor activity. Additionally, 4.5 % have a *TP53* mutation in the absence of 17p deletion (Zenz et al., 2008). Patients with a 17p deletion or *TP53* mutation are resistant to standard chemotherapy and have the most unfavorable prognosis with a median treatment free survival of 9 months and a median overall survival of 32 months (Dohner et al., 2000; Zenz et al., 2008).



Dohner et al., 2000

Figure 1.4 Probability of overall survival from time of diagnosis in patients from 5 genetic risk categories

Whole genome sequencing and whole exome sequencing has recently identified recurrently mutated genes that had not previously been associated with an adverse prognosis; these include *SF3B1*, *NOTCH1* and *BIRC3* (Fabbri et al., 2011; Puente et al., 2011; Quesada et al., 2011; Rossi et al., 2011; Wang et al., 2011). A recent multi-centre collaboration performed by the European Research Initiative in CLL sought to further define the prognostic significance of these recurrent mutations in patients with CLL by correlating presence of mutations with clinical characteristics in 3490 early stage patients. They found that *TP53*, *NOTCH-1* and *SF3B-1* mutations were associated with a shorter time to first treatment. *SF3B1* mutations were found to increase with time from diagnosis and, along with *TP53* mutations, were found to confer a poor prognosis independent of *IgV_H* status (Baliakas et al., 2015). Whole genome and whole exome sequencing currently remains in the research domain but is likely to be introduced into clinical practice in the near future. *TP53* sequencing is currently available as a diagnostic test for patients who do not have a 17p deletion by FISH in the pre-treatment setting.

1.3.4 Treatment

Treatment is reserved for patients with symptomatic, progressive disease with indications for first and second line treatment clearly defined by the International Working Group in CLL (Hallek et al., 2008). It has been demonstrated that patients with early stage, asymptomatic disease do not benefit from early treatment with alkylating agents and these patients should undergo a period of active surveillance (Dighiero et al., 1998).

Treatment options include chemotherapy and chemo-immunotherapy based regimes that can be challenging to deliver to elderly patients and to those with co-morbidities. However, the introduction of novel targeted therapies that are generally well tolerated, has provided a viable treatment option to patients considered unfit for conventional chemotherapy. The choice of treatment regimen takes into account the patient's physical fitness, presence of co-morbidities, and any previous response to therapy. It is essential to rule out a *TP53* abnormality; in the absence of a 17p deletion by fluorescence *in-situ* hybridisation (FISH), Sanger sequencing should be performed to rule out a mutation (Dohner et al., 1995). Patients with *TP53* abnormalities are refractory to standard chemotherapy and should be treated with targeted therapies.

The German CLL Study Group has categorised patients into three broad groups according to fitness for treatment; 'Go-Go' includes physically fit patients as defined by a low Cumulative Illness Rating Score and a normal renal function, suitable for standard therapy; 'Slow-Go' refers to patients with impaired physical fitness suitable for less toxic therapies; and 'No-Go' is reserved for patients with severely impaired physical fitness, best managed with symptom control (Hallek, 2013).

In all cases, patients should be considered for entry into clinical trials. The gold-standard of treatment for previously untreated 'Go-Go' patients is currently Fludarabine, Cyclophosphamide and Rituximab (FCR) which affords an overall response rate (ORR) rate of 90 %, a 5 year progression free survival (PFS) of 46.8 % and a 5 year overall survival (OS) of 78.7 % (Hallek et al., 2010). This regime is particularly effective in patients with mutated *IgV_H* genes, affording a PFS of 66.6 % at 5 years compared with 33.1 % with unmutated *IgV_H* genes (Fischer et al., 2016). However, due

to the incidence of prolonged Common Toxicity Criteria scale (CTC) grade 3-4 neutropenias within 12 months of completion of chemoimmunotherapy, this regime may be considered too toxic for older patients and those with co-morbidities.

The CLL10 study performed by the German CLL study group recently explored whether Bendamustine + Rituximab (BR) was non-inferior to FCR in the treatment-naïve setting for fit patients. They reported that although there were fewer complete responses in the BR arm, there was no survival benefit of FCR due to the increased toxicity over BR. They reported that severe neutropenia was more prevalent with FCR (84 versus 59 %) leading to an increased risk of severe infections (39 % versus 25 %) (Eichhorst et al., 2016). In the UK, Bendamustine is approved in the front line setting for patients deemed unfit for FCR.

‘Slow-Go’ treatment-naïve patients are currently treated with Chlorambucil in combination with Obinutuzumab, a Type II glycoengineered anti-CD20 monoclonal antibody. This regimen has recently been demonstrated to afford a median PFS of 26.2 months versus 15.4 months with Chlorambucil and Rituximab in treatment naïve patients with co-morbidities or renal insufficiency (Goede et al., 2015). Overall survival was improved with Chlorambucil plus Obinutuzumab versus Chlorambucil alone (hazard ratio for death, 0.41; 95 % CI, 0.23 to 0.74; $p=0.002$)(Goede et al., 2014).

The treatment of CLL is currently undergoing a revolution, with the introduction of novel targeted therapies that appear to be highly effective and are associated with minimal toxicity. Drugs that target components of the B cell receptor-signalling pathway include ibrutinib, ACP-196 (Bruton’s Tyrosine Kinase (BTK) Inhibitors), and idelalisib, an inhibitor of the delta isoform of phosphatidylinositol 3-kinase (PI3K δ).

Ibrutinib irreversibly inactivates BTK by forming a covalent bond with cysteine-481 in the active site. On a cellular level, this leads to reduced phosphorylation of ERK and PLC γ 2 and reduced nuclear expression of NF κ B Herman, 2014 (Herman, Niemann, et al., 2014b). Clinically, there is rapid and dramatic resolution of lymphadenopathy with a concurrent lymphocytosis. The observation that there is an abrupt increase in Ki67+ CLL cells in the peripheral blood, suggests that the lymphocytosis is caused by a release of recently proliferated CLL cells from the lymph-node into the peripheral blood

(Herman, Niemann, et al., 2014b). The lymphocytosis is prolonged (persists for > 12 months) in a sub-set of patients, typically those with mutated *IgV_H* genes (Woyach, Smucker, et al., 2014c). It has been reported that the prolonged lymphocytes are characterised by constitutive phosphorylation of ERK and NFκB, reduced responsiveness to IgM stimulation and reduced Ki-67 expression; all hallmarks of anergy (Woyach, Smucker, et al., 2014c). Furthermore, patients who exhibit a prolonged lymphocytosis do not have an inferior clinical outcome, reflecting that this is not a characteristic of treatment failure (Woyach, Bojnik, et al., 2014a).

In the relapsed/ refractory setting, ibrutinib affords a PFS of 88 % at 6 months and an OS of 90 % at 12 months (Byrd, Brown, O'Brien, Barrientos, Kay, Reddy, et al., 2014a). Strikingly, in patients with relapsed CLL harbouring a 17p deletion, PFS at 26 months was 56 % (Byrd, Jones, Woyach, Johnson, & Flynn, 2014b); this is in contrast to a median PFS of 11.8 months in patients with *TP53* deletion treated with high dose methylprednisolone and alemtuzumab (anti-CD56 monoclonal antibody), the previous gold standard of therapy for this indication (Pettitt et al., 2012).

In addition to the FDA approval for ibrutinib use in the relapsed/refractory and 17p-deletion settings, ibrutinib has recently been FDA approved in the treatment-naïve setting. This follows a large multicenter study of elderly, less-fit patients were randomized to receive chlorambucil or ibrutinib; patients who received single agent ibrutinib had an 18 month PFS of 94 % compared with 45 % with chlorambucil (Byrd, Jones, Woyach, Johnson, & Flynn, 2014b). Toxicity is limited, most adverse events being Grade I-II. Diarrhoea was the commonest adverse event, but it was rarely grade III-IV. Petechiae, predominantly grade I-II, affected 14 % patients and was thought to be due to inhibition of TEC kinases in platelets. Anti-coagulation with warfarin is contraindicated for patients in the UK following a small number of cases of fatal intracranial haemorrhage in the phase II trial of ibrutinib. Furthermore, it is recommended that ibrutinib should be held for three days pre- and seven days post major surgery to reduce peri-operative bleeding complications.

Ibrutinib is currently given continuously; reasons for stopping include intolerance or disease-progression. Whole exome sequencing of the first six patients to relapse on ibrutinib identified cysteine to serine mutations (C481S) in the BTK ibrutinib binding

domain in five patients, and in PLCy2, immediately downstream of BTK in the sixth patient (Woyach, Furman, et al., 2014b). Functional analysis has revealed that the BTK mutation confers reduced binding efficacy whilst the PLCy2 mutation leads to gain of function irrespective of BTK inhibition (T.-M. Liu et al., 2015; Woyach, Furman, et al., 2014b). Analysis of sequential samples suggests that these mutations were acquired during therapy and that expansion of the sub-clone harbouring the mutation pre-dates clinical relapse, theoretically providing a window to intervene before clinical progression. To date, cases of resistance to Ibrutinib have been limited to patients with 17p- or complex cytogenetics.

Idelalisib, in combination with rituximab, has been approved by the FDA for the treatment of relapsed/ refractory CLL and treatment-naïve CLL with *TP53* abnormality. Approval was granted following the landmark trial that compared rituximab with idelalisib or placebo for the treatment of patients unfit to receive standard chemotherapy. Median PFS was 5.5 months with rituximab + placebo and was not reached with rituximab + idelalisib (Furman et al., 2014). Serious adverse events that have been reported since FDA approval include hepatotoxicity, colitis, intestinal perforation and pneumonitis. The second licensed indication, for treatment-naïve patient with *TP53* abnormality is in response to a phase 2 study of idelalisib + rituximab in the elderly; nine patients harboured a *TP53* abnormality and all were alive at 3 years (O'Brien et al., 2015).

Drugs that promote apoptosis by mimicking physiological BCL-2 antagonists are particularly attractive for the treatment of CLL, in which BCL-2 overexpression is a key pathological feature. Venetoclax, a small molecule that directly targets BCL-2 is showing particular promise in early phase clinical trials for patients with poor-risk relapsed/refractory disease. Initial studies were limited by cases of fatal tumour-lysis syndrome that mandated a cautious strategy of dose escalation followed by expansion phases. PFS at 15 months was 66 % for the entire cohort and patients with 17p- had a median PFS of 16 months. 20 % patients achieved a CR and 5 % achieved MRD negativity (Roberts et al., 2016). Venetoclax has now been approved by the FDA for the treatment of relapsed CLL with 17p deletion and there is optimism that it may be a viable treatment option for patients who become resistant to BCR antagonists.

Lenalidomide, an immunomodulatory agent has shown activity as a single agent and is also undergoing investigation in the clinical trial setting. A phase 2 study in patients with relapsed/ refractory disease recently tested three starting doses of 5mg, 10mg and 15mg followed by dose escalation by 5mg every 28 days up to a maximum dose of 25mg. The ORR was 40 %, 88 % of whom received a dose \geq 15 mg/day. The average daiy dose appeared to be higher in responders. PFS was 21 months. Toxicities included neutropenia and thrombocytopenia which were independent of dose. There was a 12% risk of second primary malignancy.

In summary, it is a hugely exciting time in CLL therapeutics. We have been presented with an armory of new drugs, which we are tasked with applying safely, effectively and in a financially viable manner. These strategies will be determined by investigating the novel agents in multi-centre phase three trials in close collaboration with laboratory research.

1.4 Targeting the T Cell Component of the Tumour Microenvironment

In light of the similarities between early stage CLL and autoimmune disease, and in view of the role of T cells in contributing to the survival and proliferation of leukaemic B cells in the lymph-node, we questioned whether suppression of T cell activation could inhibit the pathogenesis of CLL. CLL T cells bear a number of phenotypic and functional abnormalities including a preponderance of effector memory cells (Nunes et al., 2012), evidence of chronic activation including PD-1 expression and shortened telomeres (Röth et al., 2008), and a restricted T cell repertoire (Farace et al., 1994) (Serrano et al., 1997) that are suggestive of an autologous antigen-driven process (Yallop, manuscript in preparation). Hypothetically, inhibition of proliferation in patients with poor-prognosis disease could delay disease progression and inhibit telomere shortening, genomic instability and clonal evolution that contribute to resistance to chemotherapy. Furthermore, by targeting the microenvironment rather than the neoplastic clone, this strategy would be unlikely to drive therapeutic resistance.

There are many drugs that have been developed to inhibit T cell activation and the most widely used in clinical practice is ciclosporin (CsA), a fungal-derived polypeptide, that was initially discovered as a potent T cell immunosuppressant in 1972 (Borel et al., 1976). CsA was found to successfully inhibit renal transplant rejection in dogs in 1977 (Calne, 1979) before being used in a clinical setting in 1978 (Calne, 1978), where it transformed the practice of organ transplantation. In addition to its ongoing use in transplantation, CsA is an effective T cell immunosuppressant in a wide-range of autoimmune and inflammatory conditions.

The effective use of CsA in the treatment of pure red cell aplasia (PRCA) complicating CLL was first reported by Chikkappa et al in 1987 (Chikkappa, Pasquale, Phillips, Mangan, & Tsan, 1987). They reported that the combined use of glucocorticoids and CsA not only achieved a remission from the PRCA but that there was a significant reduction in lymphocyte count, lymph-node and spleen size and degree of bone marrow infiltration. Similar responses have been reported by other investigators in the setting of PRCA and in the treatment of autoimmune haemolytic anaemia and immune thrombocytopenia complicating CLL. In a case series of 31 patients treated

with CsA for AIHA and ITP secondary to CLL, 5 patients were reported to have a significant reduction in the lymphocyte count from a median of $47 \times 10^9/\text{L}$ to $15 \times 10^9/\text{L}$ with a variable duration of response (Cortes et al., 2001).

The anti-tumour mechanism of action of CsA in CLL has not been fully elucidated. It has been clearly established that CsA suppresses T cell activation, differentiation and proliferation by forming a complex with cyclophilin that inhibits the capacity of calcineurin to dephosphorylate NFAT, as illustrated in Figure 1.5. NFAT is a family of five transcription factors (NFAT1-5) that share a highly conserved DNA binding domain (Macian, 2005). Dephosphorylation is required for nuclear translocation of NFAT, following which it binds to upstream enhancer elements leading to the transcription of IL2, IL4, IL10, IFN γ and TNF α (Flanagan, Corthésy, Bram, & Crabtree, 1991). Secondly, CsA inhibits the nuclear translocation of NF κ B, a family of transcription factors that share highly conserved DNA binding and dimerisation domains (Nishiyama et al., 2005). In the inactive state, the nuclear localisation domain of NF κ B-p50/ NF κ B-p65 is concealed as a result of binding to inhibitory kappa B (I κ B); activation leads to phosphorylation and ubiquitination of I κ B, leading to nuclear localisation and transcription of IL2, IL12 and IFN γ . CsA inhibits proteasomal degradation of I κ B, inhibiting nuclear translocation of NF κ B-p50/ NF κ B-p65 (Marienfeld et al., 1997; Meyer, Kohler, & Joly, 1997). Inhibition of NF κ B translocation is particularly relevant in CLL, where it is thought to mediate the pro-survival effect of CD40 ligation (Furman et al., 2000).

Furthermore, CsA inhibits the expression of CD40L and IL21 by activated CD4 $^{+}$ T cells (Fuleihan et al., 1994; Kim, Korn, Gamero, & Leonard, 2005) that normally functions to stimulate the activation, differentiation, maturation, survival and proliferation of B cells (Lederman et al., 1992; Noelle et al., 1992).

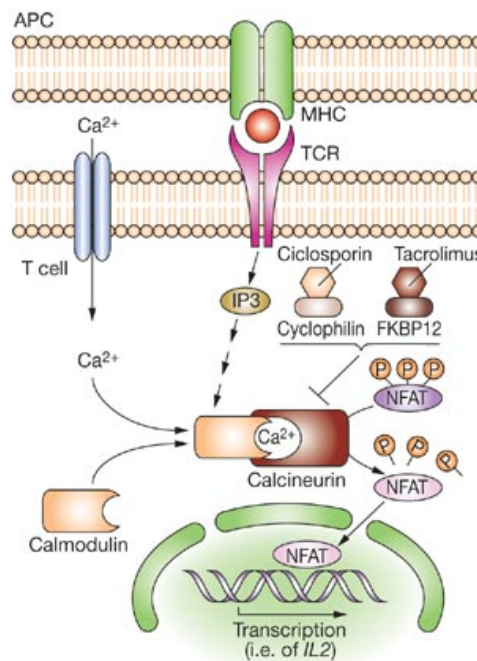


Figure 1.5 A schematic of the mechanism of action of Cyclosporin

Following TCR engagement, there is an influx of calcium that binds with calmodulin. The calcium-calmodulin complex activates the phosphatase action of calcineurin, leading to the dephosphorylation of NFAT. In the dephosphorylated state, NFAT translocates to the nucleus and activates the transcription of IL2 etc. CsA enters the cell and binds with cyclophilin, inhibiting the phosphatase activity of calcineurin.

In addition to its effects on T cells, CsA has been demonstrated to directly inhibit nuclear translocation of NFAT in IgM stimulated and CD40L+IL4 stimulated murine B lymphocytes (Choi, Brines, Holman, & Klaus, 1994; Venkataraman et al., 1994). However, investigators who have studied the differential pathways activated via IgM and CD40L stimulation of murine B lymphocytes reported that whilst IgM induced proliferation was sensitive to CsA, CD40L induced proliferation was relatively resistant (Wortis, Teutsch, Higer, Zheng, & Parker, 1995). This finding is particularly relevant to the theory that activated CD4⁺ T cells expressing CD40L play a key role in driving the proliferation of CLL cells. It suggests that any effect of CsA on CD40 signalling in CLL cells is likely to be due to down-regulation of CD40L expression by CD4 T cells rather than inhibition of signalling downstream of CD40 in CLL cells.

In-vitro studies of the effect of CsA in CLL are limited in number. Schmid et al demonstrated that IL2 and TNF α driven proliferation of highly purified CLL cells was sensitive to CsA in a dose-dependent manner (Schmid, Merk, & Porzsozt, 1994). However, it remains unanswered whether IgM, CD40L or activated T cell-stimulated CLL cells are directly sensitive to CsA.

Manipulation of the tumour microenvironment using CsA to inhibit T cell-induced CLL survival and proliferation is a novel approach to the management of patients with adverse prognostic factors. Patients with early-stage, asymptomatic CD38⁺ CLL, destined to have an inferior overall survival (Damle et al., 1999; Hamblin et al., 2002) are currently understudied and would potentially benefit from an alternative strategy to 'watchful waiting'. We therefore opted to investigate the novel strategy of targeting the microenvironment to inhibit CLL proliferation in this cohort of patients. We set up a Phase II clinical trial (CyCLLe) with the support of Bloodwise (previously known as Leukaemia and Lymphoma Research). The primary outcome of this study was change in CLL proliferation rate after four weeks of CsA therapy. Secondary outcomes included toxicity and disease response rates. The plan was to proceed to a larger cohort Phase 3 study with clinical outcome measures if CyCLLe demonstrated a significant change in proliferation.

In preparation for the *in-vivo* study, the precise mechanism of action of CsA on CLL and CLL T cells was investigated in a series of *in-vitro* studies. We started by investigating whether CLL T cells are indeed sensitive to the effect of CsA. Having established that CLL T cells are sensitive to the effect of CsA, we went on to investigate whether CsA has an additional, direct effect on CLL cells, independent of any T cell mediated effect. As it has been shown that CD40L signalling plays a major role in T cell mediated activation and proliferation of CLL cells in the lymph-node (Ghia et al., 2002; Patten, Buggins, Richards, Wotherspoon, Salisbury, Mufti, Hamblin, & Devereux, 2008b), we investigated if CsA has a direct effect on CD40L activated CLL cells. We subsequently investigated the effect of CsA on the interaction between CLL and activated autologous CD4 T cells, aiming to discern whether the effect in CLL cells is direct, indirect (T cell dependent), or a combination of the two. The effect of CsA on NFAT and NF κ B activation in CLL and CD4 T cells was performed to further define its mechanism of action.

In view of the role of NFAT and NFκB in mediating BCR signalling and in maintaining a state of anergy in CLL, we then went on to investigate whether CLL cells activated through the BCR are directly sensitive to CsA. As it has been shown that inhibition of NFAT signalling reverses anergy and stimulates apoptosis in CLL with features of anergy (Apollonio et al., 2013), we assessed the effect of CsA on the viability of CLL cells. The *in-vitro* studies were subsequently applied to patients recruited to the clinical trial to further investigate the effect of CsA in *in-vivo*.

The proliferation of CLL cells was measured *in-vivo* using labelling with deuterated glucose, an entirely novel method of investigating the effect of a therapeutic agent. Since transit between lymph-node and tissues is considered to play a role in disease pathogenesis, the kinetics of release and disappearance of proliferating cells was measured before and after treatment with CsA.

A second clinical study, '*In-vivo* Kinetics in Chronic Lymphocytic Leukaemia' was initiated to formally study the degree of intra-patient variation in leukaemic cell proliferation rates. Although it would have been preferable to measure intra-patient variation in proliferation rates before commencing CsA, this was deemed too burdensome to mandate within the trial protocol and was therefore considered an optional investigation. This observational study additionally provided the opportunity to intensively investigate the kinetics of CLL proliferation and trafficking between the lymph-node and peripheral blood compartments.

In addition to studying the CLL population as a whole, we investigated the kinetics of sub-populations defined by a phenotype previously associated with trafficking (CXCR4/CD5) and sIgM expression, previously associated with proliferation. Finally, the kinetics of subpopulations defined by BCR internalisation was investigated in order to establish the relationship between proliferation and a functional parameter related to CLL biology.

We investigated the relationship between the various sub-populations by performing serial sampling of labelled CLL cells and by comparing the degree of labelling in each sub-population at regular intervals over an eight-week period.

1.5 Aims and Objectives

1. To dissect the mechanism of action of CsA in CLL using *in-vitro* methods:-

- To investigate and fully characterise the direct effect of CsA on CLL B and CD4 T cells.
- To investigate if T cell suppression using CsA indirectly inhibits CLL cell activation.

2. To investigate whether manipulation of the tumour microenvironment using CsA is a safe and effective method of inhibiting proliferation in patients with early-stage, adverse-risk disease:-

- To investigate the safety and efficacy of CsA therapy in patients with poor-prognosis (defined by CD38 expression) that does not meet conventional criteria for treatment.
- To use *in-vivo* deuterium labelling as a novel method of measuring the effect of drug on tumour proliferation.
- To apply *in-vitro* assays to further define the effect of CsA *in-vivo*.

3. To use *in-vivo* labelling of CLL cells to investigate the kinetics of proliferation and trafficking of CLL cells:-

- To measure *in-vivo* proliferation rates and to investigate intra-patient variation in proliferation rates.
- To investigate release and disappearance rates of recently proliferated cells.
- To investigate the relationship between sIgM expression, BCR internalisation and proliferation and kinetics of release and disappearance.

Chapter 2 . General Materials and Methods

2.1 Primary Patient Material

2.1.1 Ethical Approval

Peripheral blood mononuclear cells (PBMCs) were collected from randomly selected patients with a confirmed diagnosis of CLL. Ethical approval was provided by a National Research Ethics Service (NRES reference 08/H0906/94) and informed written consent was obtained according to the Declaration of Helsinki. Blood was donated by healthy donors for use as 'normal controls'.

2.1.2 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by density-gradient separation using Histopaque 1077 (Sigma Aldrich). The cells were centrifuged for 25 minutes at 400 *g* with no brake. The monolayer was harvested and washed in phosphate buffered saline (PBS) before being pelleted for 10 minutes at 300 *g*. Platelets were removed by resuspending the pellet in PBS and centrifuging at 300 *g* for 10 minutes twice. Manual cell counting was performed using a counting chamber (Hawksley), with trypan blue solution as a viability stain.

2.1.3 CLL cell culture

CLL cells were cultured in Roswell Park Memorial Institute Medium (RPMI 1640, Sigma), 10 % (v/v) Foetal Bovine Serum (FBS, Sigma), L-Glutamine (2mM final), penicillin (2000 units per mL) and streptomycin (2mg/mL) Complete medium (CM). All cell culture was performed at 37°C, 100 % humidity and 5 % CO₂.

2.1.4 Fibroblast culture

Untransfected fibroblasts and fibroblasts transfected with CD40L were used (gift from Prof Pepper, Cardiff University). Fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with glucose (4500 ng/l), L-Glutamine, sodium pyruvate and sodium bicarbonate supplemented with 10 % (v/v) FBS, penicillin (2000 units per mL) and streptomycin (2mg/mL). Fibroblasts were cultured at an initial concentration of 2×10^5 /ml in 24 well plates and left for 12 hours to adhere to the plastic and to achieve confluence. Cells were passaged by washing twice with PBS and incubating with trypsin-EDTA for 2 minutes to allow cell detachment before washing with 50mL PBS containing 10 % (v/v) FBS.

2.1.5 Freezing cells

PBMCs were frozen at 10^6 - 10^7 cells per mL in 50 % CM and 50 % freezing medium (containing 80% (v/v) FBS and 20 % (v/v) dimethyl sulphoxide (DMSO)). Cells were aliquoted into cryovials (Nalgene) and immediately transferred to a Mr. Frosty™ freezing container that contained 70 % (v/v) isopropanol. This was placed in a -80°C freezer and left over night to allow controlled freezing. The following day, cryovials were transferred to a liquid nitrogen containing storage facility for long-term storage within King's College London Human Tissue Authority Approved (HTA) Tissue Bank (licence 12223, September 2006, unconditional status).

2.1.6 Reviving cells

On collection from the cryostore, cryovials were rapidly thawed in a 37°C water bath and washed in PBS to remove the DMSO-containing freezing medium before re-suspending in pre-warmed complete media.

2.1.7 T cell isolation

T cells were negatively selected from PBMCs using an EasySep™ T cell separation kit (Stem Cell) as per the manufacturer's instructions. PBMCs were resuspended at 5×10^7 in PBS containing 2 % (v/v) FBS and labelled with tetrameric antibody complexes recognizing CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, glycophorin A and dextran-coated magnetic particles. The labelled cells were separated using an EasySep™ magnet and the unbound T cells poured off into a new tube.

CD4+ T cells were selected using the same EasySep™ technology but with a kit (Stem Cell) using tetrameric antibody complexes that additionally recognised CD8 and TCR γ/δ .

2.1.8 CLL cell isolation – negative selection

CLL cells were negatively selected using an EasySep™ B cell separation kit without CD43 (Stem Cell) as per manufacturer's instructions. Unwanted cells were targeted for removal with tetrameric antibody complexes recognizing CD2, CD3, CD14, CD16, CD56, glycophorin A and dextran-coated magnetic particles. The labelled cells were separated using the EasySep™ magnet and the unbound CLL B cells harvested.

2.2 Activation Methods

2.2.1 T cell activation using Phorbol 12-Myristate 13-Acetate (PMA) and Ionomycin

T Cells were activated using PMA (Sigma) at 10 ng/mL and Ionomycin (Sigma) at 1 μ /mL at a concentration of 1×10^6 cells/mL in CM in 24 well plates at 37°C in a fully humidified atmosphere of 5 % CO₂.

2.2.2 T cell activation using anti-CD3/CD28

24 well plates were coated with 500 μL of agonistic anti-CD3 (ebiosciences) at 2.5 $\mu\text{L}/\text{mL}$ in PBS (Sigma). The antibody was allowed to bind to the plastic at 4°C for 24 hours. The unbound antibody-containing solution was completely aspirated and wells were gently washed with PBS before addition of the T cell suspension (1×10^6 cells/ml in CM). Anti-CD28 (ebiosciences) was added to the suspension at 1 $\mu\text{L}/\text{mL}$). Cells were activated at 37°C in a fully humidified atmosphere of 5 % CO_2 .

2.2.3 B cell stimulation using anti-IgM

Normal and CLL B cells were stimulated through the B Cell Receptor (BCR) using goat F (ab')₂ anti-human IgM (μ chain specific) (Southern Biotech) at a concentration of 10 $\mu\text{g}/\text{mL}$. B cells were activated at $1 - 2 \times 10^6 / \text{mL}$ in CM at 37°C in a fully humidified atmosphere of 5 % CO_2 .

2.2.4 B cell stimulation using CD40L transfected fibroblasts

CD40L transfected fibroblasts were allowed to adhere to plastic (24 well plate) and were confirmed by light microscopy to be confluent before the extraction of DMEM and addition of purified CLL cells at a concentration of $1 \times 10^6 / \text{mL}$ in CM. Cells were co-cultured at 37°C in a fully humidified atmosphere of 5 % CO_2 for 24 hours before mechanical detachment of CLL cells.

2.3. Reagents

2.3.1 Ciclosporin for *in-vitro* use.

Ciclosporin (Sigma) was dissolved in ethanol to make a stock concentration of 1 mg/mL and stored in aliquots at -20°C. A working concentration of 10 µg/mL was prepared for same-day use. A final concentration of 200 ng/mL was applied to all experiments.

Table 2.1 General Consumables

Consumable	Manufacturer
7- Amino-Actinomycin (7-AAD)	BD Biosciences
Annexin V	BD Biosciences
Annexin V Binding Buffer	BD Biosciences
Anti Human CD3	eBioscience
Anti Human CD28	eBioscience
BD Cytofix/Cytoperm™	BD Biosciences
Bovine Serum Albumin	Sigma Aldrich
Brefeldin	BD Biosciences
Bromophenol Blue	Sigma Aldrich
BD Perm/Wash Buffer™	BD Biosciences
BD CompBeads anti-mouse Igk	BD Biosciences
4',5-diamidino-2-phenylindole (DAPI)	Sigma
Dimethyl Sulphoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
Dulbecco's Modified Eagle Medium	Sigma
ECL Western Blotting Detection Reagent	GElifesciences
EasySep Negative Human CD3 Kit	Stemcell
EasySep Negative Human CD4 Kit	Stemcell
EasySep Negative Human CD19 Kit (without CD43)	Stemcell
Ethanol	Fischer Scientific
Ethylenediaminetetraaceticacid (EDTA)	ThermoFischer
EZ-Run™ Pre-stained Rec Protein Ladder	ThermoFischer
Fixable Viability Dye eFluor® 780	eBiosciences
Foetal Calf Serum	Sigma
Glycerol	Sigma
Goat F(ab') ₂ Anti-Human IgM (μ chain specific)	SouthernBiotech
Histopaque 1077	Sigma Aldrich
Human HT-12 v4.0 Expression BeadChip Kit	Illumina
Ionomycin	Sigma Aldrich

L-glutamine	Sigma Aldrich
Methanol	ThermoFischer
1 % NP-40	Sigma Aldrich
Paraformaldehyde 16 % w/v aq sol	VWR
Phorbol 12-myristate 13-acetate	Sigma Aldrich
Phosphate buffered saline	Sigma Aldrich
Phosphatase Inhibitor Cocktail II, III	Sigma Aldrich
Protease Inhibitor Cocktail	Sigma Aldrich
R6K screen tapes	Agilent technologies
R6K reagents	Agilent technologies
Red cell lysis buffer	eBioscience
RNAeasy [®] mini kit	Qiagen
Roswell Park Memorial Institute (RPMI) 1640	Sigma Aldrich
0.1% Sodium Azide	Severn Biotech
0.1% sodium deoxycholate	Sigma Aldrich
Sodium dodecyl sulfate (SDS)	Sigma Aldrich
SuperScript [®] III Reverse Transcriptase	Life Technologies
TargetAmp [™] Nano-g [™] Biotin-aRNA Labelling Kit	Cambio/Epicentre
Tris HCl	Sigma
0.1 % Triton X-100	Sigma Aldrich
Trypan Blue	Fluka Analytical
Trypsin	Sigma Aldrich
Tween-20	Sigma

2.4 Flow Cytometry

2.4.1 Preparation of cells for assessment of surface antigens

Cells were washed twice in PBS and re-suspended at $1-2 \times 10^5$ in 1 mL PBS before adding 1 μ L fixable viability dye (Ebiosciences). Cells were incubated with the dye for 10 mins at room temperature in the dark before washing twice with PBS. Cells were then re-suspended at $1-2 \times 10^5$ in 100 μ L PBS incubating with saturating concentrations of the relevant antibodies for 20 minutes at 4°C in the dark. Cells were washed twice in PBS before analysis on the flow cytometer (BD, FACSCanto II).

2.4.2 Preparation of cells for analysis of intracellular cytokines

Brefeldin (Sigma) 1 μ L /mL was added to the activation wells 6 hours before termination of the activation period. Brefeldin is a fungal metabolite that disrupts the golgi apparatus and prevents the secretion of cytokines so that they are retained within the cytoplasm. Cells were then washed and stained with fixable viability dye and for cell surface antigens as above. Fixation was subsequently performed using 400 μ L Cytofix per test (BD Cytofix™) for 10 minutes. Cells were then washed and resuspended in 1 mL permeabilisation buffer (BD Perm/Wash Buffer™) for 15 minutes at room temperature. The cells were pelleted at 300 *g* for 5 minutes and supernatant was aspirated. Cells were resuspended in 100 μ L permeabilisation buffer and incubated with the antibody to the intracellular cytokine for 20 minutes at room temperature. Cells were washed twice in permeabilisation buffer before analysis on the flow cytometer (BD, FACSCanto II).

2.4.3 Apoptosis Assay

Viability assays were performed using Annexin V-conjugated to FITC and 7-AAD (BD Biosciences). Annexin V binds to phosphatidyl serine, which is present on the external cellular surface early in the process of apoptosis. 7-AAD is a cell-impermeant dye that is able to bind ds-DNA in cells where the cellular membrane has been compromised (as in fully apoptotic cells). 100 µL Annexin V binding buffer (BD Biosciences) was added to the experimental sample with 2.5 µL Annexin-V antibody + 1 µL 7-AAD and allowed to incubate for 15 minute in the dark at room temperature. 400 µL Annexin V binding buffer was then added prior to sample analysis by flow cytometry.

2.4.4 Compensation Matrix for Flow Cytometry

Compensation beads (BD Biosciences) were used to create the compensation matrix for fluorochromes conjugated to surface antigens. Positive and negative beads were vortexed for 30 s before adding one droplet to 100 µL PBS and incubating with 5 µL fluorochrome-conjugated antibody for 20 minutes in the dark at 4°C. Beads were washed twice before re-suspending in 100 µL PBS for acquisition on the flow cytometer.

Live/ Dead cells were used to create the viability compensation control. Fresh PBMCs were prepared at a concentration of $1 \times 10^6/\text{mL}$ and equally divided into 2 round-bottomed FACS tubes. One tube was placed in a 65°C water bath for 4 minutes and then on ice for 5 minutes. Live and dead cells were then recombined and stained with the fixable viability dye as above before washing twice for acquisition on the flow cytometer.

20,000 events were acquired for each compensation control and the matrix was calculated using BD FACS DIVA software.

2.4.5 Controls

Fluorescence minus one controls were prepared for fluorescence gating of surface antigens. Isotype matched controls were used for intracellular fluorescence gating.

2.4.6 BD FACSCanto II (BD Biosciences) Lasers and Fluorochromes

Data was acquired using the BD Biosciences FACSCanto II, configured as in Table 2.2.

Flow cytometry antibodies and their fluorochromes are listed in Table 2.3.

Table 2.2 BD FACSCanto II configuration

Lasers	Violet	405 nm solid state, 30mW laser	
	Argon (Blue)	488 nm solid state, 20mW laser	
	Red	633 nm HeNe, 17mW laser	
Detectors	8 PMTs in 4-2-2 configuration		
Fluorochromes	Violet	Pacific Blue AmCyan	450nm 488nm
	Blue	FITC PE PerCP/-Cy5.5 Pe-Cy-7	525nm 575nm 678/ 695nm 785 nm
	Red	APC APC-Cy7	660nm 785 nm
Detector bands	Violet	450/50; 502-525 nm	
	Blue	530/30; 585/42; >670 nm	
	Red	660/20; 780/60 nm	

Table 2.3 Flow Cytometry Antibodies

Antibody	Clone	Supplier
CD3 - V500	UCHT1	BD
CD3 – PB	HIT39	Biolegend
CD4 – ef450	OKT4	Biolegend
CD4 – PercP-Cy5.5	OKT4	Biolegend
CD5 – FITC	UCHT2	Biolegend
CD5 – PE-Cy7	UCHT2	Biolegend
CD8 – PercP-Cy5.5	SK1	Biolegend
CD8 – APC	SK1	Biolegend
CD5 – PE-Cy7	UCHT2	Biolegend
CD19 – PE	HIB19	Biolegend
CD19 – APC	HIB19	Biolegend
CD25 – APC	BC96	Biolegend
CD38 – PE	HIT2	Biolegend
CD45-RA	HI100	Biolegend
CD69 – AF488	FN50	Biolegend
CD69 – PE	FN50	Biolegend
CD154 (CD40L) – PE	24-31	Biolegend
CD279 (PD1) – APC	EH12.2H7	Biolegend
IFN γ – BV421	4S.3B	Biolegend
CXCR4 – PE	12GS	Biolegend

2.5 Image Flow-Cytometry - Amnis ImageStream X[®] (Merck)

Image flow cytometry was performed using the Amnis ImageStream X[®] which combines cellular microscopy (40 x magnification lens) with flow-cytometry. By collecting large numbers of digital images per sample and by providing numerical representation of image-based features, individual cell information is combined with statistical significance afforded by large sample sizes.

2.5.1 Configuration

The ImageStream X[®] has six co-linear lasers and a 40 x magnification lens allowing 6 images (bright field plus 5 x fluorescent channels) to be collected for each cell to be collected simultaneously with a high resolution camera. Figure 2.1 demonstrates how the ImageStream X[®] works and laser configuration is shown in Table 2.4.

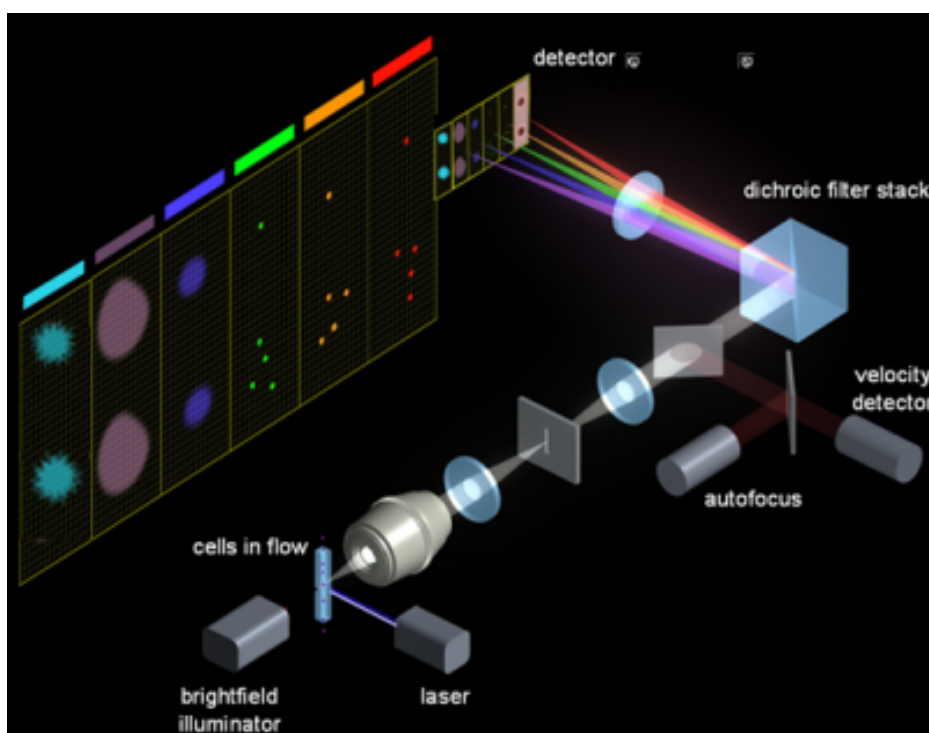


Figure 2.1 ImageStream X[®] Configuration

Hydrodynamically focused cells are trans-illuminated by a brightfield light source and orthogonally by lasers. An objective lens collects fluorescence emissions, scattered and transmitted light from the cells. The collected light intersects with the spectral decomposition element. Light of different spectral bands leaves the decomposition element at different angles such that each band is focused onto 6 different physical locations of the camera. The camera operates in TDI (time delay integration) mode that electronically tracks moving objects.

Table 2.4 ImageStream X® Fluorochromes and Detection Channels

		Excitation Laser					
Camera 1		405nm	488nm	561nm	592nm	658nm	785nm
	Channel 1 430-505nm	DAPI, Hoechst					
	Channel 2 505-560nm		FITC, AF488				
	Channel 3 560-595nm		PE	PE, AF456			
	Channel 4 595-660nm		PE-TexRed	AF568	TexRed, AF495		
	Channel 5 660-745nm		PE-Cy5	PE-Cy5	AF647	AF647, AF660, AF680	
	Channel 6 745-800nm		PE-Cy7	PE-Cy7	APC-Cy7	APC-Cy7	SSC

2.5.2 Preparation of cells for Image Flow Cytometry

1-3 x 10⁶ cells were collected, washed in ice-cold PBS/2 % v/v FBS and pelleted in a pre-cooled centrifuge for 5 minutes at 300 g. Cells were surface-stained on ice and washed in ice-cold PBS 2 % v/v FBS containing 1mM EDTA. Cells were then fixed in 400 µL 4 % paraformaldehyde (VWR) for 10 minutes. The fixed cells were then washed in PBS and re-suspended in 100 µL permeabilisation buffer (containing 2 % FBS, 0.1 % v/v Triton X-100 (Sigma), 0.1 % v/v sodium azide (Severn Biotech). The primary antibody was added to the permeabilisation buffer at a concentration of 10 µg/mL and was allowed to incubate at room temperature for 20 minutes. Cells were washed once in permeabilisation buffer and resuspended at 100 µL with the secondary antibody at a concentration of 15 µg/mL. This was allowed to incubate for 15 minutes before the cells were washed twice with PBS/2 % v/v FBS. A minimum sample size of 60 µL at a cellular concentration of 1 x 10⁶/mL was prepared. DAPI (1:10 v/v) (Sigma) was added immediately before analysis.

Antibodies used in this investigation are listed in Table 2.5.

Table 2.5 Image Flow Cytometry Antibodies

Antibody	Dilution	Source
CD4 – PercpCy5.5	5:100	Biolegend
CD19 – PE	5:100	Biolegend
Rabbit anti-NFAT-C2	1:20	Santa Cruz
Rabbit anti-NFκB-p65	1:20	Santa Cruz
F(ab') ₂ Donkey anti-rabbit IgG-FITC	1:200	Jackson ImmunoResearch

2.5.3 Compensation and controls

The compensation matrix was produced using fresh primary cells that had been prepared as single stained controls. Fluorescence minus one controls were used for fluorescence gating. Imaging controls were prepared using an unstimulated sample (untranslocated). A sample stained for the secondary antibody only was used as a further staining control.

2.5.4 Image Analysis using IDEAS® software

ImageStream Data Exploration and Analysis Software (IDEAS®) was used to calculate the percentage translocation of the transcription NFAT and NFκB-p65 in CLL and CD4 T cells. After compensation, similarity analysis was performed on single, focused cells based on their width to height ratio and high nuclear contrast (measured by the gradient max feature). The degree of nuclear translocation was measured by comparing the nuclear fluorescence image with the pattern of fluorescence produced by the nuclear label. Nuclear translocation is considered to have occurred when there is an overlap between nucleus and transcription factor signal. The similarity score is calculated from the Pearson's correlation coefficient based on a linear regression analysis of pairs of values taken from different data sources.

Nuclear to cytoplasmic ratio of transcription factor signal was calculated by creating a 'mask' around the nucleus, defined by the DAPI signal followed by a single pixel erosion to ensure that no cytoplasmic signal was included. The cytoplasmic mask was

created by subtracting the nuclear mask from the transcription factor mask. The ratio of the transcription factor integral in the nucleus compared to that integral in the cytoplasmic mask was used to create this feature.

An example of the gating strategy is shown in Figure 2.2.

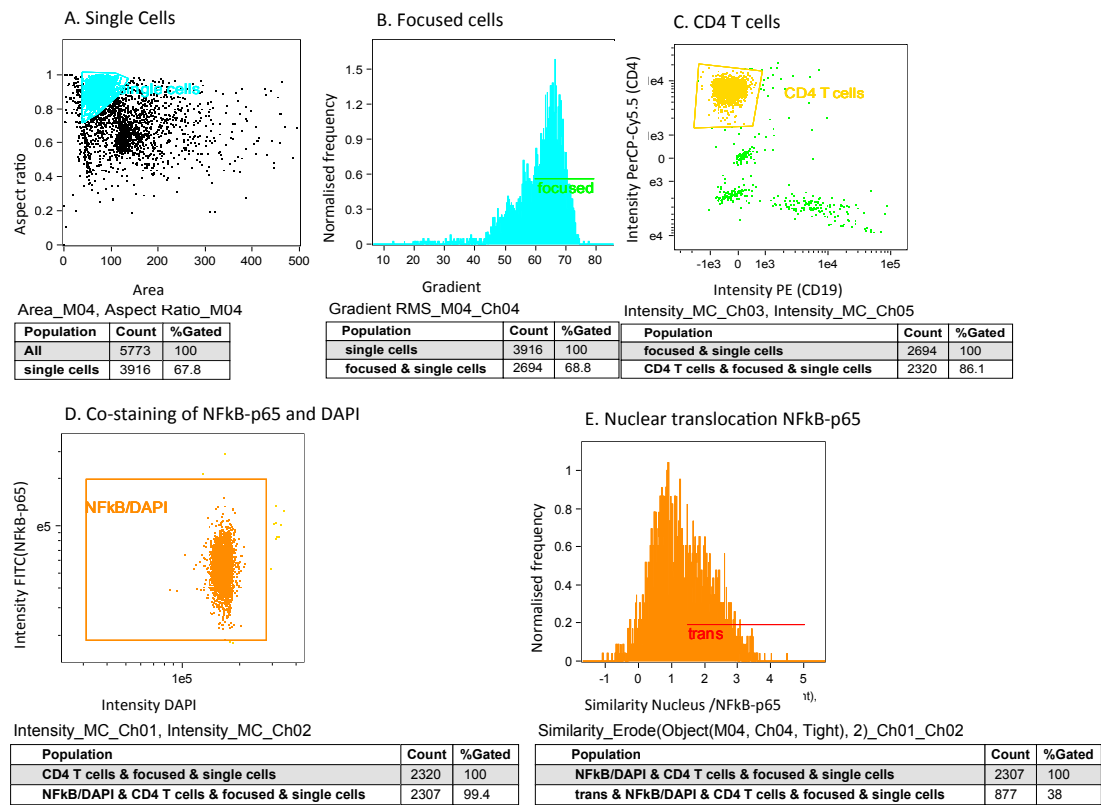


Figure 2.2 (a-e) Example of gating strategy using IDEAS® to assess NFκB-p65 translocation

Single, focused cells expressing CD4 were gated on, followed by cells that co-expressed of NFκB-p65 and DAPI. The degree of nuclear translocation was calculated by measuring the similarity between the nuclear (DAPI) and NFκB-p65 signal.

2.6 Western Blotting for ERK and Phosho-ERK

2.6.1 Solutions and Reagents

Lysis buffer

- 125mM Tris HCl pH 6.8
- 4 % w/v SDS
- 40 % v/v glycerol
- 200mM DTT
- 0.002 % w/v bromophenol blue

2.6.2 Cell Lysis

Cell lysates were prepared by adding 50 μ L ice-cold lysis buffer to 1×10^6 cells (dry pellets). Following mechanical disaggregation, lysates were heated to 95-100°C for 5 minutes and then briefly centrifuged (30 s, 1000x G_{\max}). The tube was flicked to ensure complete mixing.

2.6.3 Protein Electrophoresis and Western Blotting

Whole cell lysates were separated by Polyacrylamide Gel Electrophoresis (PAGE) using the NuPAGE system (Invitrogen). Protein lysates of between 1×10^5 and 3×10^5 were loaded per lane on a 4-1 % (w/v) bis-Tris polyacrylamide gel. EZ-Run™ pre-stained Rec protein ladder (Thermo Fischer) was used to determine the molecular weight of the bands. Gels were assembled in a NuPAGE gel apparatus and run for 1 hour in 1 x Running Buffer (Invitrogen: 50mM MES, 50mM Tris, 0.1 % w/v SDS, 1 nM EDTA, pH 7.3) at 200V. After electrophoresis, the separated proteins were transferred to a nitrocellulose-coated nylon membrane (Hybond c-Extra, GE healthcare) using the NuPAGE semi-dry gel blotting module according to the manufacturer's instructions. Transfer was for 1.5 hours in NuPAGE Transfer buffer (Invitrogen: 25mM Bicine, 25mM Bis-Tris, 1 mM EDTA, pH 7.2) with 20 % v/v methanol at 25V.

2.6.4 Antibody Staining

Membranes were washed x 2 in PBS- 0.05 % Tween and blocked for one hour with BSA- PBS 0.05 % Tween (3 % w/v) or non-fat dried milk-PBS Tween (10 % w/v) prior to washing x 3 with PBS-0.05 % Tween and overnight incubation at 4°C with primary goat-anti human antibodies to phospho-ERK (in BSA- PBS 0.05 % Tween, 1:1000 v/v) and total ERK (in non-fat dried milk-PBS 0.05 % Tween, 1:1000 v/v) (Cell Signalling). Following washing x 3 in PBS- 0.05 % Tween, horseradish peroxidase (HRP)-conjugated rabbit-anti-goat antibody 1:2000 v/v (Dako) was incubated for 1 hour before washing x 3 in PBS- 0.05 % Tween.

Enhanced chemo-luminescence (ECL™, GE lifesciences), consisting of luminol and hydrogen peroxide solution was used to visualize the bands (HRP cleaves the luminol agent to produce light). Detection was performed using photographic film (Hyperfilm ECL, GE Healthcare), which was developed using a Compact X4 X-ray developer (Konica Minolta).

Densitometric analysis of ERK-specific bands was performed with ImageJ Software (GE Healthcare). The values of individual patients were calculated as percentage of the positive control and determined as the ratio of the optical density of phospho-ERK and optical density of total ERK.

2.7 Gene Expression Studies

RNA was prepared from purified CD4 and CD8 T cells. Purification was performed using fluorescence activated cell sorting and purity was confirmed to be ≥ 95 % by flow cytometry. Cells were temporarily stored at -80°C and RNA was prepared in batches using the RNeasy® Mini kit (Qiagen) as per manufacturer's instructions. The concentration and quality of the RNA was assessed using the NanoDrop 2000 spectrophotometer (ThermoFischer Scientific) before being transferred on dry ice for analysis of gene expression at the Biomedical Research Centre, Guy's Hospital.

Gene expression studies were performed using Illumina® iScan technology. Briefly, cDNA was generated from mRNA in the sample using reverse transcription. The cDNA was then transcribed into cRNA, which was biotin-labelled, amplified and purified and quantified. The cRNA was then allowed to hybridise to the bead chip (Human HT-12 v4.0 Expression BeadChip, Illumina®) and was washed and stained with streptavidin-Cy3. The bead chip was then scanned using the Illumina® iScan and data analysed using Genome Studio to assess the genes expressed in the samples.

2.8 Data Analysis

Data was analysed using GraphPad v. 7.0 (Prism). Significance was assessed using either a Student's paired t test or a Wilcoxon matched-pairs signed rank test according to whether the data passed the D'Agostino & Pearson normality test.

Chapter 3 . *In-Vitro* Studies of the Effect of Ciclosporin A on Healthy Donor and CLL T and B Cells

3.1 Background and Rationale

The main objective of this study was to determine whether manipulation of the tumour microenvironment using ciclosporin A (CsA) to inhibit T cell activation, could be applied as a therapeutic strategy in early stage, adverse risk CLL. It has previously been shown that CLL cells become activated and undergo proliferation in the lymph-node compartment where interaction with activated CD4 T cells is thought to promote tumourigenesis (Herishanu et al., 2011; O. Jaksic, Kardum Skelin, & Jaksic, 2010; Pascutti et al., 2013; Patten, Buggins, Richards, Wotherspoon, Salisbury, Mufti, Hamblin, & Devereux, 2008b). Patten *et al* demonstrated that within the CLL lymph-node, recently proliferated CLL cells were closely associated with activated CD4 T cells and that co-culture of CLL cells with CD4 T cells induced the activation and proliferation of CLL cells (Patten, Buggins, Richards, Wotherspoon, Salisbury, Mufti, Hamblin, & Devereux, 2008b). Furthermore, Bagnara *et al* demonstrated that activated CD4 T cells were necessary for the engraftment, survival, and proliferation of CLL cells in the non-obese diabetes/severe combined immunodeficiency/ $\gamma\text{C}^{\text{null}}$ (NSG) mouse model (Bagnara et al., 2011). These studies suggest that targeting CD4 T cell activation could indirectly suppress CLL cell activation and proliferation.

In preparation for the clinical trial investigating the effect of CsA on CLL proliferation *in-vivo*, the effect of CsA on healthy donor and CLL T cells was studied. As CsA is known to inhibit NFAT and NF κ B activation, and in view of the role of these transcription factors in mediating B Cell Receptor (BCR) signalling in CLL, the direct effect of CsA on CLL cells was also investigated. Finally, an *in-vitro* co-culture system was developed to simulate the effect of CsA on CLL cell: CD4 T cell interactions in the tumour-microenvironment.

Early *in-vitro* studies into the mechanism of action of CsA suggested that it has a T lymphocyte specific effect. Borel *et al* demonstrated that CsA suppressed the proliferation of concanavalin (ConA) stimulated splenic T lymphocytes (ConA is a lectin that cross links the T cell receptor) but that it did not inhibit proliferation of

lipopolysaccharide (LPS) stimulated B lymphocytes in nude mice (Borel et al., 1977). In agreement with this theory, White et al demonstrated that a dose of 0.08-1 µg/mL CsA was sufficient to inhibit phytohaemagglutinin stimulated T lymphocytes (PHA, another lectin that activates T cells by cross-linking the T cell receptor), whereas doses of at least 5 µg/mL CsA were required to inhibit proliferation of B lymphocytes induced by sheep anti-porcine IgM (White et al., 1979).

Subsequent investigations into the mechanism of action of CsA revealed that in T cells stimulated using ConA or anti-CD3, interleukin 2 (IL2) production/ responsiveness and cellular proliferation were strongly inhibited in the presence of the drug (Bunjes et al., 1981). However, T cells stimulated with anti-CD28 (co-stimulation) in addition to anti-CD3 were found to be less sensitive to the effect of CsA, whilst cells stimulated with anti-CD28 alone were found to be resistant (June et al., 1987). As anti-CD28 activation differs from anti-CD3 activation by stimulating cyclic guanosine monophosphate (cGMP) signalling rather than calcium signalling, these observations suggested that CsA targeted the calcium-dependent pathway.

The effect of CsA on B lymphocytes was also found to depend on the mechanism of stimulation. B cell proliferation induced by anti-IgM, which activates calcium signalling, has been shown to be sensitive to CsA whilst LPS, which stimulates cells via a calcium-independent mechanism, has been demonstrated to be resistant to CsA (Choi et al., 1994; Fuleihan et al., 1994; Klaus, 1988). The observation that B cell proliferation induced by CD40L, either alone or expressed by activated T cells is relatively resistant to the effect of CsA, suggests that there are further qualitative differences between methods of activation that determine drug sensitivity (Kim et al., 2005; K. Schuh, Avots, Tony, Serfling, & Kneitz, 1996; Wortis et al., 1995).

In 1991, Flanagan *et al* reported that CsA inhibits calcium-dependent T cell activation by forming a complex with a prolyl isomerase, preventing the nuclear translocation of NFAT (Flanagan et al., 1991; Le Roy et al., 2012; K. Schuh et al., 1996). Later studies confirmed that CsA binds with high affinity to the cyclophilins which possess peptidyl-proline-cis-trans isomerase activity (Schreiber, 1991), forming a complex that inhibits the calcium and calmodulin dependent activity of calcineurin (J. Liu et al., 1991). Inhibition of calcineurin inhibits its phosphatase activity, which is required for the

dephosphorylation of NFAT - a process that, on TCR ligation, enables its translocation from the cytoplasm to the nucleus. Within the nucleus, NFAT proteins initiate the transcription of a number of genes including IL2, IL4, IL6, IL21 and CD40L (Fuleihan et al., 1994; Kim et al., 2005) .

In addition to its effects on NFAT in T cells, CsA has been demonstrated to inhibit members of the NF κ B transcription factor family including NF κ B-p65, -p50 and c-rel in T cells by inhibiting the degradation of I κ B, a necessary step for NF κ B nuclear translocation (Marienfeld et al., 1997). CsA has also been found to inhibit the JNK and p38 mitogen-activated protein kinase (MAPK) pathways that are further upstream and essential for T cell activation (Hewamana et al., 2009; Su et al., 1994).

Although CsA has been found to inhibit NFAT translocation in B lymphocytes activated by anti-IgM, phorbol 12-myristate 13-acetate (PMA) and Ionomycin (Venkataraman et al., 1994) and CD40L /IL4 (Choi et al., 1994; Flanagan et al., 1991; K. Schuh et al., 1996), there is no evidence in the literature that CsA inhibits NF κ B translocation in B lymphocytes activated by any of these mechanisms.

NFAT and NF κ B have been shown to play a key role in CLL pathogenesis but the effect of CsA on these transcription factors has not previously been investigated in CLL. In all cases studied, levels of NFAT-C1 and NFAT-C2 are increased in CLL cells compared with healthy B lymphocytes (Le Roy et al., 2012; K. Schuh et al., 1996). NFAT-C2 levels have been found to be comparable with those of T lymphocytes and furthermore, have been found to be highest in those patients that do not respond to signalling through the BCR (Le Roy et al., 2012).

NFAT-C1 levels are under the transcriptional control of NFAT-C2 (Zhou et al., 2002) and as NFAT-C1 has been shown to play a key role in B lymphocyte survival, has been a greater focus of studies of BCR signalling in CLL. Apollonio *et al* have reported that constitutive NFAT-C1 nuclear localisation is a key characteristic of CLL cells that have an 'anergic' phenotype i.e. express low levels of sIgM, do not mobilise calcium in response to sIgM ligation and exhibit constitutive ERK phosphorylation. Inhibition of NFAT-C1 nuclear translocation using the cell-permeable VIVIT peptide that competes with calcineurin for NFAT binding restores BCR signalling capacity and in doing so,

impairs CLL cell survival (Apollonio et al., 2013). Although Le Roy *et al* found that NFAT-C1 was constitutively activated in CLL regardless of BCR signalling capacity; they found that nuclear localisation could be induced upon BCR ligation in those cases considered to be 'responders'. They found that NFAT-C1 translocation in 'responders' could be blocked by the VIVIT peptide and that this inhibited metabolic activity (Le Roy et al., 2012). In the MEC1-transplanted mouse model of CLL (characterised by an anergic phenotype), injection of the VIVIT peptide reduced tumour volume and increased survival providing further evidence for the role of NFAT in CLL pathogenesis (Apollonio et al., 2013). The cause of NFAT up-regulation in CLL remains unknown, however it is possible that it is driven by antigen ligation of the BCR *in-vivo*.

The NFκB family of transcription factors has also been demonstrated to play a major role in CLL pathogenesis. The NFκB family is comprised of p50, p52, p65 (Rel-A), c-Rel, and Rel-B components, which, in the inactive state, exist as cytoplasmic homodimeric or heterodimeric complexes, bound to IκB proteins. Upon stimulation, IκB is phosphorylated by IκB Kinase (IKK) and subsequently ubiquitinated and degraded, allowing translocation of NFκB to the nucleus and transcription of NFκB target genes (Huxford, Huang, Malek, & Ghosh, 1998). Pro-survival, anti-apoptotic gene products related to NFκB activation include BCL-2, Bcl-X_L, and Survivin and FLICE inhibitory protein (FLIP) (Cuní et al., 2004; Granziero et al., 2001).

In CLL, it has been found that there is constitutive activation of NFκB and furthermore, that increased basal NFκB-p65 - DNA binding is predictive of advanced stage, high tumour burden and short time to first treatment (Furman et al., 2000; Hewamana et al., 2009). Nuclear translocation of NFκB-p65 in CLL cells can be induced by CD40 ligation, by sIgM ligation and as a consequence of BAFF and APRIL signalling in the tumour microenvironment (Endo et al., 2007; Furman et al., 2000; Hewamana et al., 2008). NFκB-p65 translocation is disrupted by LC-1, a parthenolide derivative that inhibits IKK and this leads to reduced viability of CLL cells (Hewamana et al., 2008). Although the effect of CsA on NFκB-p65 activation has not been studied in CLL cells, the observation that CsA disrupts NFκB-p65 activation in T cells suggests that it may have a direct effect on CLL cell survival (Marienfeld et al., 1997; Meyer et al., 1997).

In-vitro studies into the effect of CsA in CLL are limited in number. Schmid *et al* demonstrated that IL2 and TNF α driven proliferation of highly purified CLL cells was sensitive to CsA in a dose-dependent manner (Schmid *et al.*, 1994). However, the effect of CsA on transcription factor activation in CLL cells warrants further investigation.

The observation that in 20 % of patients treated with CsA for autoimmune-complications of CLL, there is an anti-leukaemia effect, reflects that CsA is active in a proportion of patients with this disease (Cortes *et al.*, 2001). However, the nature of the *in-vivo* effect may be a direct effect on CLL cells, an indirect T cell dependent effect or a combination of the two.

To address this, a series of *in-vitro* assays was developed to investigate the effect of CsA on CLL cells, CD4 T cells and the interaction between the CLL and activated autologous CD4 T cells.

We started by investigating the effect of CsA on CLL derived T cells; although the effect of CsA on healthy donor T cells is well documented, the effect on CLL T cells has not been reported. Secondly, the effect of CsA on the ability of CLL cells to respond to T cell activation was investigated. Activation was designed to reproduce lymph-node based events and therefore included CD40L activation to simulate CD4- T cell mediated activation of CLL cells.

We then simultaneously investigated both the direct and indirect, T cell dependent effects of CsA on CLL cells by co-culturing CLL cells with activated autologous CD4 T cells.

As BCR mediated signalling in CLL has been shown to be sensitive to the VIVIT peptide that inhibits NFAT activation (Apollonio *et al.*, 2013; Le Roy *et al.*, 2012), the effect of CsA on BCR signalling was investigated. Finally, in view of the fact that inhibition of NFAT signalling has been shown to reverse anergy and to induce apoptosis (Apollonio *et al.*, 2013), the effect of CsA on the viability of CLL cells was investigated.

To more precisely define the effect of CsA in CLL, the effect of the drug on the transcription factors NFAT-C2 and NF κ B-p65 in CD4 T cells and CLL cells was assessed.

Although NFAT-C1 activation is classically associated with B cell malignancies, NFAT-C2 was measured in CLL cells as there was no suitable NFAT-C1 antibody available for flow cytometry.

We planned to apply the *in vitro* assays developed in this investigation to further explore the *in-vivo* effect of CsA in the CyCLLe study.

3.2 Methods

Comprehensive general methods are detailed in Chapter 2.

T cells were stimulated with anti-CD3 and anti-CD28 to achieve physiological activation. A strong proliferative signal is afforded by anti-CD3 stimulation whilst anti-CD28 provides 'signal 2' to reduce the number of cells becoming anergic or undergoing apoptosis. Activation was achieved using anti-CD3 coated plates and unbound anti-CD28 rather than using bead-bound antibodies to avoid potential difficulties interpreting flow-cytometry data in the presence of beads.

T cell activation was measured using a combination of CD69, a surface marker of activation and IFN γ , a Th1 intracellular cytokine released upon T cell activation. IFN γ was measured as it has been reported to be produced by CLL-specific Th1 cells which have been shown to activate CLL cells via the induction of CD38 in a T-bet dependent fashion (Bürgler et al., 2015; Gitelson et al., 2003; Os et al., 2013).

The direct effect of CsA was measured on CLL cells activated through CD40 (CD40L transfected fibroblasts), activated autologous CD4 T cells and via BCR ligation. Activation was measured by flow-cytometric assessment of CD25 and CD69 expression. CD25 is the alpha unit of the IL2 receptor, and CD69 is a membrane protein expressed with four hours of lymphocyte activation (Simms & Ellis, 1996).

The effect of CsA on the interaction between activated CD4 T cells and CLL cells was assessed by co-culturing these cells at a ratio of 1:4, similar to that found in the CLL lymph-node compartment.

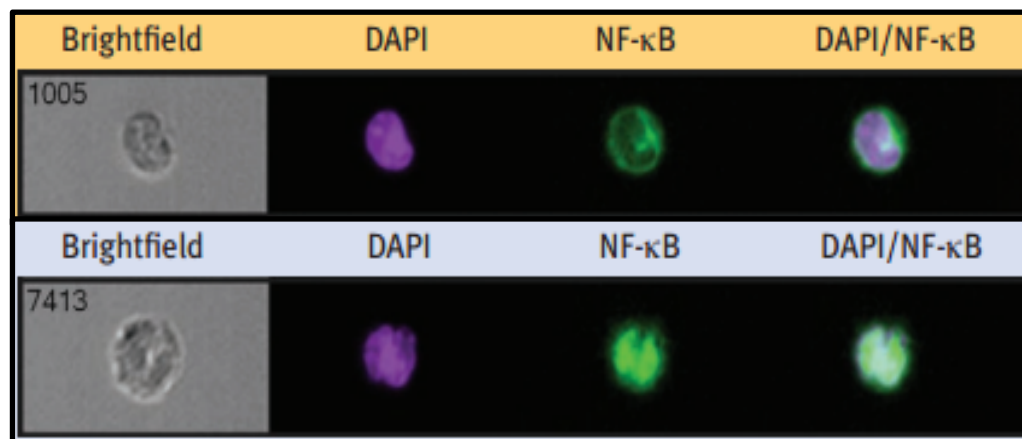
The effect of CsA on cell viability was measured flow-cytometrically using annexin-V and 7-AAD staining. Annexin-V binds to phosphatidylserine that translocates to the extra-cellular membrane at early stages of apoptosis and 7-AAD passages through disrupted cell membranes of cell at later stages of apoptosis to bind with high affinity to DNA.

The effect of CsA on NFAT and NF κ B activation in CLL cells and CD4 T cells was measured using image flow-cytometry (Amnis ImageStream X[®]), which combines

microscopy with flow-cytometry (Chapter 2.5). There are many established methods of assessing the subcellular localization of NFAT and NFκB including Western blotting, Electrophoretic Mobility Shift Assays (EMSA), Enzyme Linked Immunosorbent Assays (ELISA) and immunohistochemistry. However, image flow-cytometry allows rapid quantitative assessment of subcellular localisation of transcription factors in large numbers of cells. Furthermore, surface staining allows distinction of transcription factor localisation in different cell populations within a sample. The IDEAS® image analysis software applies features (algorithms) and masking operations (region-finders), to perform image-based analysis. To assess events of translocation, a similarity score algorithm is used. This is calculated by log transforming the Pearson's correlation coefficient for pixel values of the DAPI (nuclear) and FITC (NFκB/ NFAT) signals. Examples of non-translocated and translocated NFκB are illustrated in Figure 3.1.

Assessment of nuclear translocation was initially challenging; difficulties involved the preparation of concentrated activated cells in a single-cell suspension, optimisation of the fixation and permeabilisation protocol, maintenance of a central fluidics stream, camera-focus, laser function and software issues. The design of the activation assays required a significant amount of preparation including testing different fluorochromes, and analysing the time-course of transcription factor translocation in response to various stimuli.

a.



b.

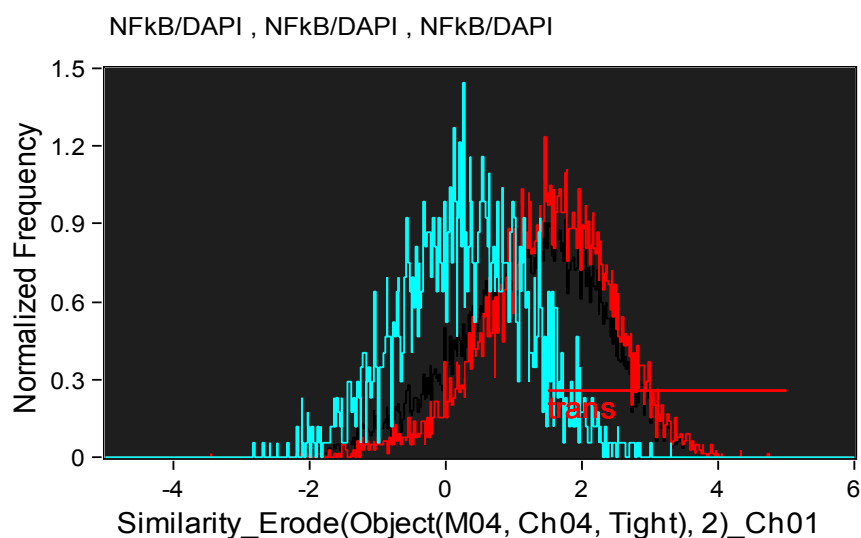


Figure 3.1 a Examples of non-translocated and translocated NFκB-p65 in CLL CD4+ T cells

Each cell is imaged (x40) shown in “Brightfield”. DAPI is used to stain the nucleus and NFκB-p65 is stained with FITC. Nuclear translocation is calculated by measuring the similarity between the nuclear and transcription factor stains. Upper row illustrates non-translocated NFκB-p65 and lower row illustrates translocated NFκB-p65.

b Exemplary histogram demonstrating degree of similarity between nucleus and NFκB-p65

The similarity between NFκB-p65 and nuclear stain is illustrated pre-activation, in blue, and post activation, in red.

3.3 Results

3.3.1 Ciclosporin A inhibits healthy donor and CLL CD4 T cell activation stimulated by anti-CD3/anti-CD28 in a dose-dependent manner

The optimum CsA concentration for *in-vitro* studies was determined by performing a series of dose-response assays, where the inhibition of expression of T cell activation markers was determined over a range of CsA concentrations. As plasma concentrations of greater than 200 ng/mL are associated with *in-vivo* toxicity, this was the maximum concentration investigated *in-vitro*. PBMCs from healthy donors and CLL patients were activated in the presence of increasing concentrations of CsA, from 10 ng/mL to 200 ng/mL. Activation status at 24 hours was determined by CD69 and IFN γ co-expression by flow-cytometry. Figure 3.2 illustrates the gating strategy applied. Figure 3.3 demonstrates that CsA inhibits healthy donor and CLL CD4 T cell activation in a dose-dependent manner with maximum inhibition occurring at 100-200 ng/mL.

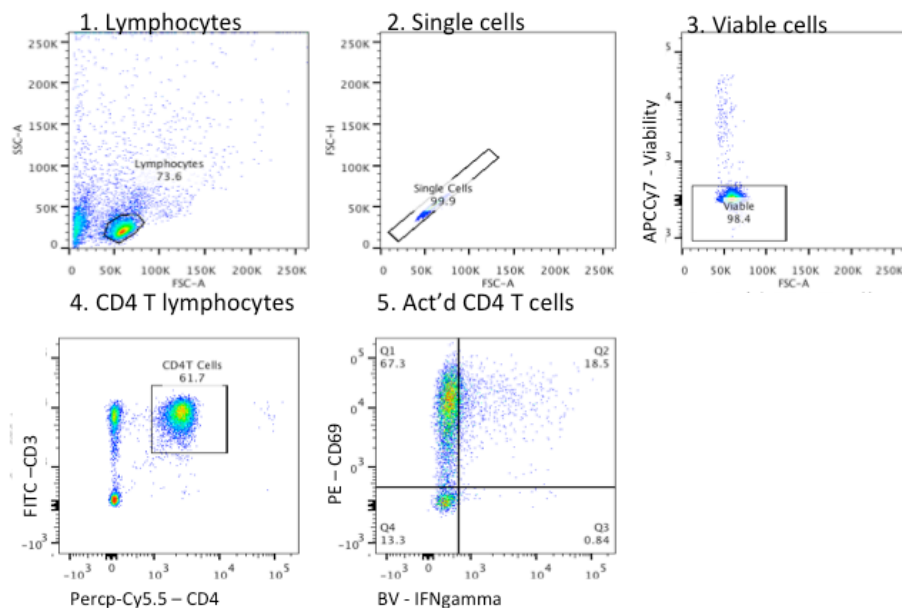


Figure 3.2 Gating strategy applied to assess activation of T cells

Flow cytometry data was acquired using the BD Canto 2. Compensation was applied and data analysed using FlowJo v10.6. Gates were set using fluorescence-minus one controls.

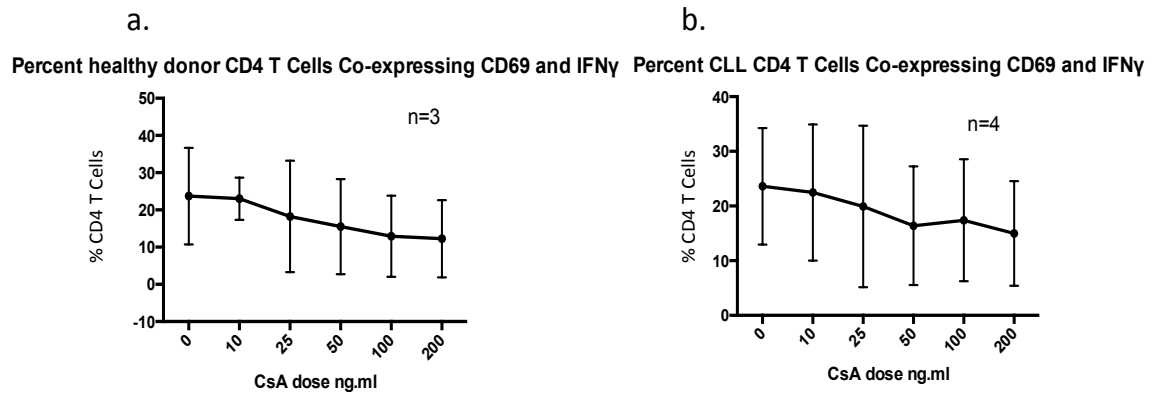


Figure 3.3 (a&b) CsA inhibits activation of healthy donor and CLL T cells in a dose-dependent manner

PBMCs were activated using anti-CD3 coated plates and unbound anti-CD28. PBMCs from healthy donors and CLL patients were activated in the presence of increasing concentrations of CsA. CD69 and IFN γ co-expression was measured at 24 hours using flow cytometry. Viable CD3 $^{+}$ /CD4 $^{+}$ cells were gated on. Results are plotted as mean with standard deviation.

3.3.2 Ciclosporin inhibits healthy donor and CLL CD4 and CD8 T cell activation stimulated by anti-CD3/anti-CD28

To investigate whether CsA could significantly overcome T cell stimulation induced by anti-CD3/anti-CD28, PBMCs from healthy donors and CLL patients were activated in the presence of CsA or a vehicle control (VC). A concentration of 200 ng/mL CsA was used as this was demonstrated to have the greatest suppressive effect in the limited dose-response assays. Activation status at 24 hours was determined by flow cytometric assessment of CD69 and IFN γ co-expression. Figure 3.4 demonstrates that in healthy donor activated CD4 and CD8 T cells, 200 ng/ml CsA inhibits co-expression of CD69 and IFN γ from a mean of 23.7 % to a mean of 8.7 % (Wilcoxon matched-pairs signed rank test $p=0.06$) and from a mean of 19.3 % to a mean of 6.3 % (Wilcoxon matched-pairs signed rank test, $p=0.06$) respectively. Co-expression of CD69 and IFN γ by activated CLL CD4 T cells was reduced from a mean of 19.8% to a mean of 6.7% (paired t-test, $p=0.003$) and by activated CLL CD8 T cells from a mean of 20% to a mean of 6.6% (paired t-test, $p=0.03$) in the presence of 200 ng/ml CsA.

Having found that CsA significantly reduces activation of healthy donor and CLL CD4 and CD8 T cell activation, it was then necessary to investigate whether CsA disrupts the mechanism whereby CD4 T cells activate CLL cells. As the evidence for T cells in CLL pathogenesis is limited to CD4 T cells, further experiments focused entirely on CLL CD4 T cells.

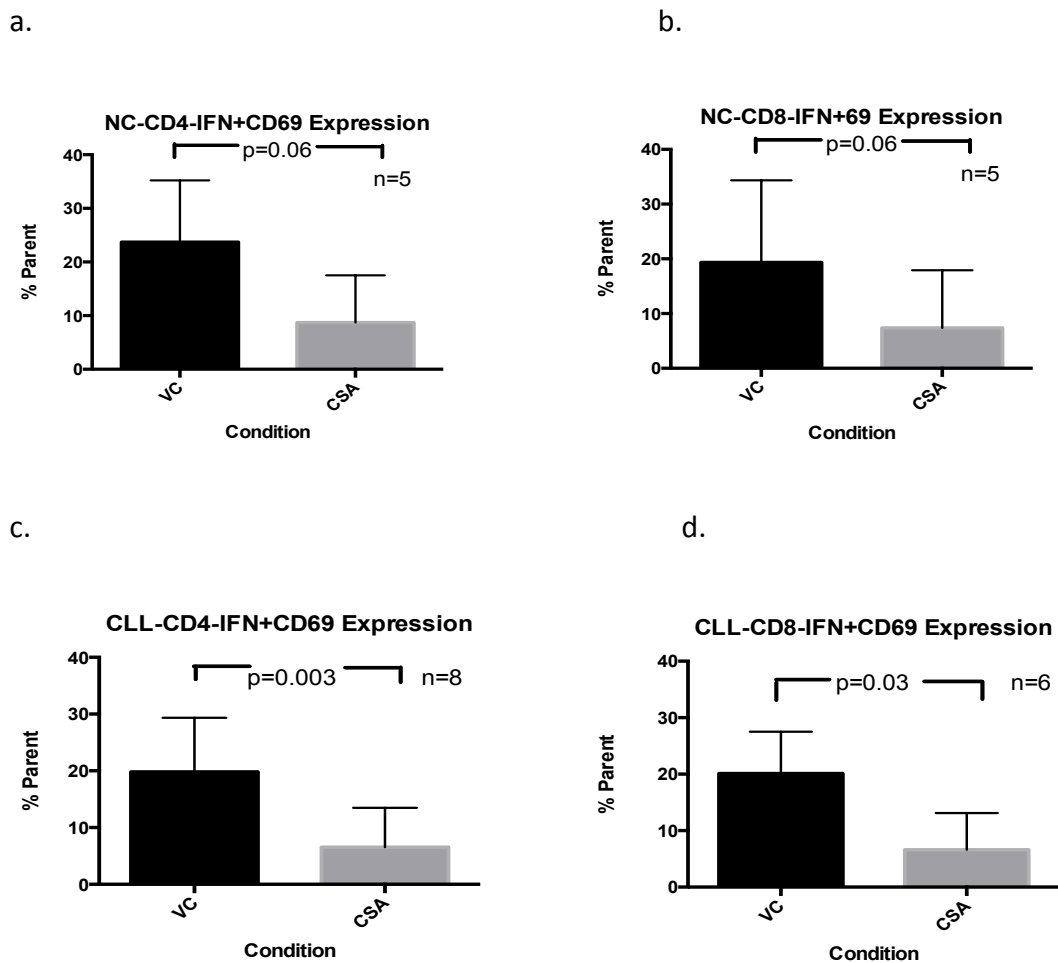


Figure 3.4 (a-d) Cyclosporin inhibits activation of normal and CLL CD4 and CD8 T Cells

PBMCs from normal donors (NC) and CLL patients (CLL) were activated using anti-CD3 coated plates and unbound anti-CD28 in the presence of CsA or vehicle control (VC). CD69/ IFN γ co-expression was measured at 24 hours using flow cytometry. Viable CD3+/CD4+ and viable CD3+/CD8+ cells were gated on (gates were set using fluorescence minus one controls). 'Percent parent' denotes the percent of T cells that express CD69 and IFN γ .

3.3.4 Ciclosporin inhibits CD40L expression in activated CLL CD4 T cells

Within the tumour microenvironment, CD40L expression and IL21 secretion are considered to form major components in the process of activation of CLL cells by CD4 T cells. IL21 is a type 1 cytokine; it is strongly induced upon TCR ligation and stimulates STAT 1 and STAT 3 signalling. The IL21 5' regulatory region contains three NFAT binding sites and induction of IL-21 promoter activity has been found to be impaired by CsA treatment (Kim et al., 2005). CD40L expression by activated T cells has previously been found to be NFAT dependent and sensitive to CsA (Fuleihan et al., 1994; Tsytsykova, Tsitsikov, & Geha, 1996).

CD40L and IL21 expression was measured after 12 and 24 hours respectively in viable CLL CD4 T cells activated with anti-CD3 and anti-CD28 in the presence of CsA 200 ng/ml (CsA) or vehicle control (VC).

Figure 3.5.a demonstrates that there was a 22 % reduction (paired t-test, $p=0.03$) in CD40L Median Fluorescence Intensity (MFI) in CLL CD4 T cells in the presence of 200 ng/ml CsA. Figure 3.5.b demonstrates that CsA did not inhibit expression of IL21 by activated CLL CD4 T cells at 24 hours.

The strong inhibition of CD40L expression by CD4 T cells suggests that this would disarm their potential to activate CLL cells in the lymph-node compartment. The absence of inhibition of IL21 secretion may be due to the high level of IL21 fluorescence in the unstimulated state (Figure 3.5c). A blocking antibody could have been applied to achieve a true negative control.

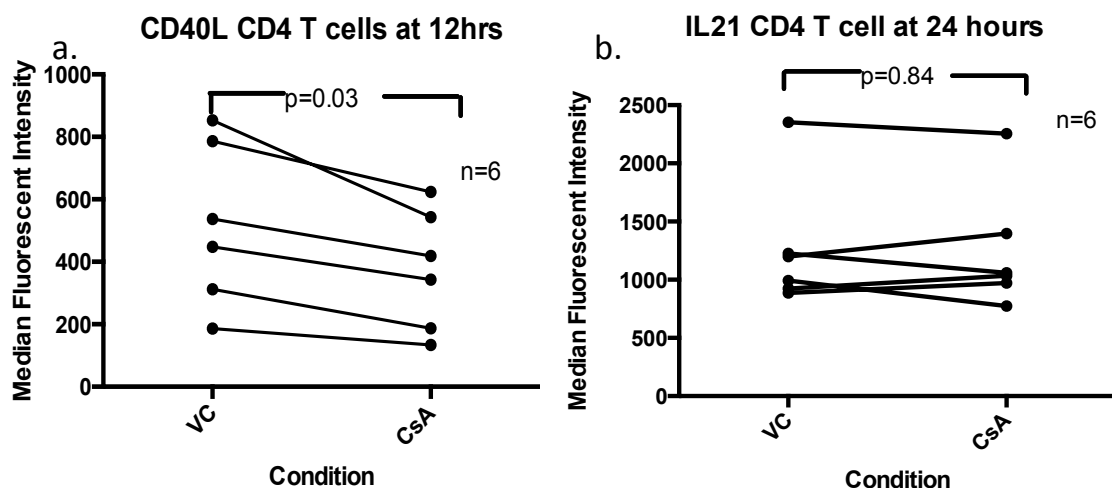


Figure 3.5 (a-b) CsA inhibits CLL CD4 T cell expression of CD40L but does not inhibit IL21 secretion

a. CLL CD4 T cells were activated using anti-CD3 coated plates and unbound anti-CD28 in the presence of CsA 200 ng/ml or vehicle control (VC). CD40L expression was measured at 12 hours using flow cytometry. b. IL21 expression was measured after 24 hours using intracellular flow-cytometry. Brefeldin was added 12 hours before the end of incubation. Viable CD3+/CD4+ cells were gated on (gates were set using fluorescence minus one controls).

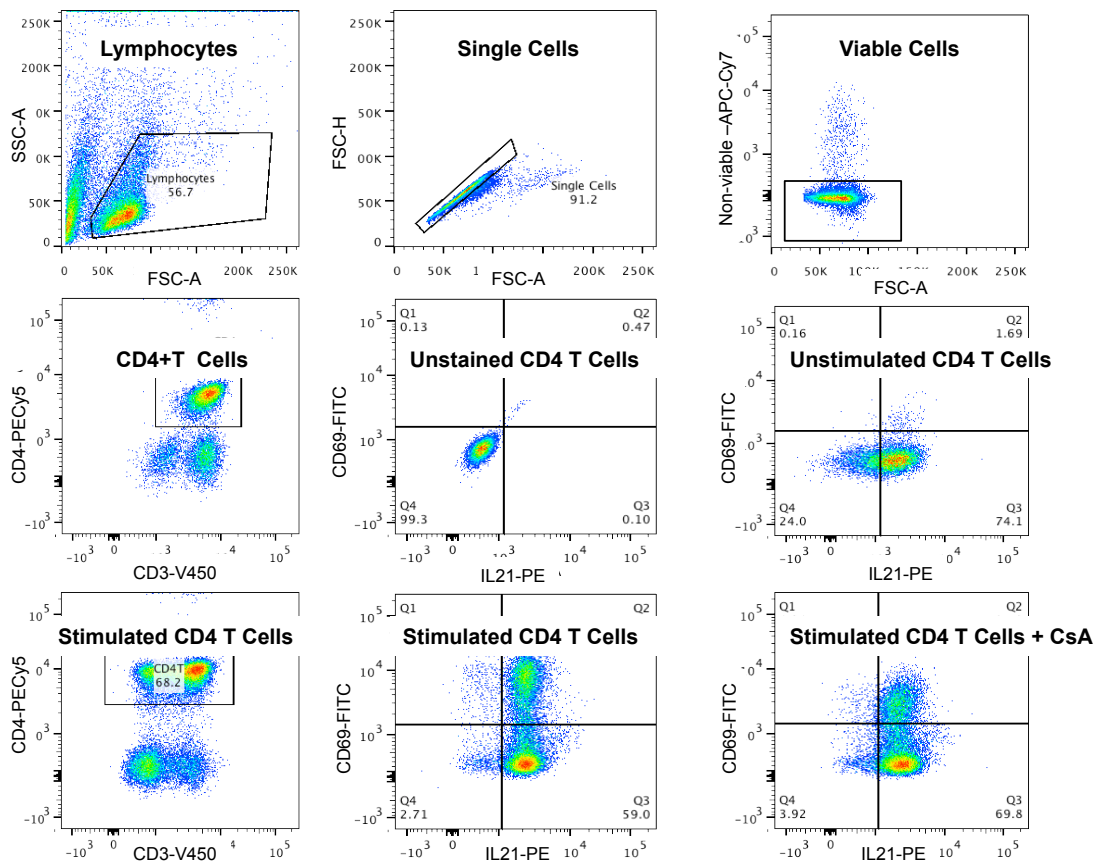


Figure 3.5 c. Gating strategy illustrating expression of IL21 by resting, stimulated and CsA-treated CLL CD4 T cells

CLL CD4 T cells were activated using anti-CD3 coated plates and unbound anti-CD28 in the presence of CsA 200 ng/ml or vehicle control (VC). IL21 expression was measured after 24 hours using intracellular flow-cytometry. Brefeldin was added 12 hours before the end of incubation. Viable CD3+/CD4+ cells were gated on (gates were set using fluorescence minus one controls).

3.3.5 Ciclosporin does not inhibit healthy donor or CLL B cell activation induced by CD40L

Having demonstrated that CsA has a suppressive effect on CLL T cells, the direct effect of CsA on healthy donor and CLL B cells was investigated. CD40L was used to activate CLL cells as this molecule is transiently expressed by activated T cells in CLL proliferation centres and is thought to play a role in CLL survival and proliferation via CD40 engagement and NF κ B activation (Furman et al., 2000; Ghia et al., 2002). CD40 is a member of the tumour necrosis factor receptor family expressed by B cells. Co-culture of CLL B cells with CD40L transfected fibroblasts has been shown to stimulate proliferation (Hamilton et al., 2012).

To investigate whether CsA can overcome CD40L induced activation of CLL cells, negatively selected healthy donor and CLL B cells were co-cultured with CD40L transfected fibroblasts in the presence of CsA 200 ng/ml (CsA) or VC. Activation was assessed after 24 hours by measuring CD25 and CD69 expression. Figure 3.6 demonstrates that CsA does not inhibit the expression of CD25 or CD69 by healthy donor B cells (n=4) or CLL cells (n=8) activated with CD40L. In contrast to CD69 expression downstream of BCR signalling, CD69 induced by CD40L was insensitive to CsA suggesting that there are significant differences in transcription factor activation according to the mechanism of CLL cell stimulation. In addition to a qualitative difference in the mechanism of stimulation, it is also possible that CD40L stimulation using CD40L transfected fibroblasts provides a greater intensity of stimulation that cannot be overcome by CsA.

Additionally CsA does not inhibit the expression of CD25 or CD69 by CLL cells co-cultured with untransfected fibroblasts (n=8) demonstrating that CsA does not directly inhibit expression of these activation markers in the resting state.

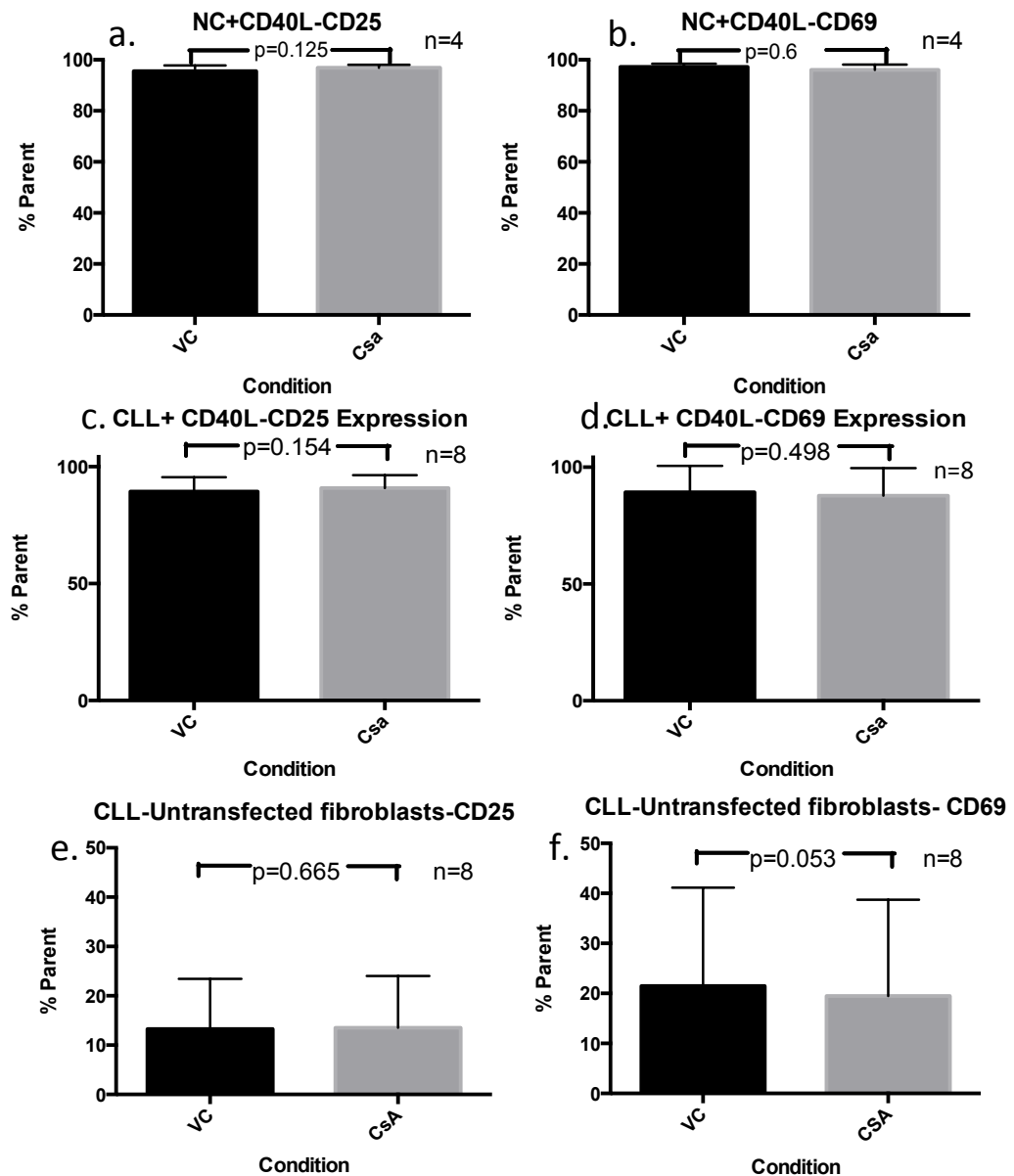


Figure 3.6 (a-f) Activation status of healthy donor (NC) and CLL B cells co-cultured with CD40L transfected and untransfected fibroblasts in the presence of CsA or vehicle control

Negatively selected B cells/CLL cells were co-cultured with fibroblasts in the presence of CsA. Expression of CD25 and CD69 was measured after 24 hours by flow cytometry. Viable cells expressing CD19 and co-expressing CD5/ CD19 were gated on to identify healthy donor and CLL cells respectively (gates were set using fluorescence minus one controls). Percent parent denotes the percentage of B/ CLL cells expressing CD25 and CD69.

3.3.6 Ciclosporin has a major CD4 T cell dependent effect and a minor direct effect on CLL cells co-cultured with autologous CD4 T cells

In view of the observation that activated autologous CD4 T cells stimulate the activation and proliferation of the tumour-cells *in-vivo* (Bagnara et al., 2011; Patten et al., 2005), and in light of the effect of CsA on CLL T cells, we went on to investigate whether CsA can inhibit CD4 T cell dependent activation of CLL cells. A system was developed whereby CLL cells were co-cultured with autologous CD4 T cells that had been activated using anti-CD3/anti-CD28 in the presence of CsA or a VC for 12 hours. To assess for any direct effect of CsA, CLL cells were negatively selected using immune-magnetic beads, conditioned with 200 ng/mL CsA / VC for one hour and washed, before being combined with the CD4 T cells for co-culture, as illustrated in Figure 3.7. Activation of CLL B and T cells was assessed daily for 3 days by flow cytometric assessment of CD25 and CD69 expression by viable CLL B cells and CD25, CD69 and CD40L expression by viable CD4 T cells.

Figure 3.8 is a representative plot demonstrating the time-course of activation of CD4 T cells and CLL cells from a single patient following co-culture. This was repeated using cells from six different patients (summarised in Figure 3.10). The results demonstrate that after activation with anti-CD3/CD28 in the presence of CsA, there is marked inhibition of T cell expression of CD25, CD69 and CD40L that is evident throughout the co-culture period. After 48 hours, in the presence of CsA, CD40L MFI was reduced by a mean of 23.5 % (Wilcoxon test, $p=0.03$). At 72 hours, there was a trend towards inhibition of CD25 expression (MFI was inhibited by a mean of 64 % (Wilcoxon test, $p=0.56$)), CD69 was reduced by a mean of 39 % (Wilcoxon test, $p=0.03$) and CD40L by a mean of 40 % (Wilcoxon test, $p=0.06$) (Figure 3.9 a-c). The lack of statistical significance of change in CD25 expression appears to be due to the heterogeneity within our sample of cases rather than absence of effect. A larger sample size would be necessary to investigate this further.

Treatment of CD4 T cells with CsA significantly impaired their ability to activate CLL cells; CD25 MFI was reduced by a mean of 64 % (Wilcoxon test, $p=0.03$) and CD69 by a mean of 63 % (Wilcoxon test, $p=0.03$) in CLL cells co-cultured with CsA-treated CD4 T cells.

In addition to the T cell dependent effect of CsA on CLL cell activation, there was a smaller direct effect; MFI of CD25 was reduced by a mean of 41 % (Wilcoxon test, $p=0.06$) and CD69 by a mean of 29 % (Wilcoxon test, $p=0.03$) at 72 hours in CsA pre-conditioned CLL cells activated with untreated autologous CD4 T cells. Inhibition of CD25 expression did not reach statistical significance.

There was a reciprocal reduction in the activation status of CD4 T cells co-cultured with CsA pre-conditioned CLL cells; expressed lower levels of CD40L (mean reduction of 7 % (Wilcoxon test, $p=0.03$)) and CD69 (mean reduction of 12 % (Wilcoxon test, $p=0.03$)), further emphasising the bi-directional nature of signalling between CLL and CD4-T Cells.

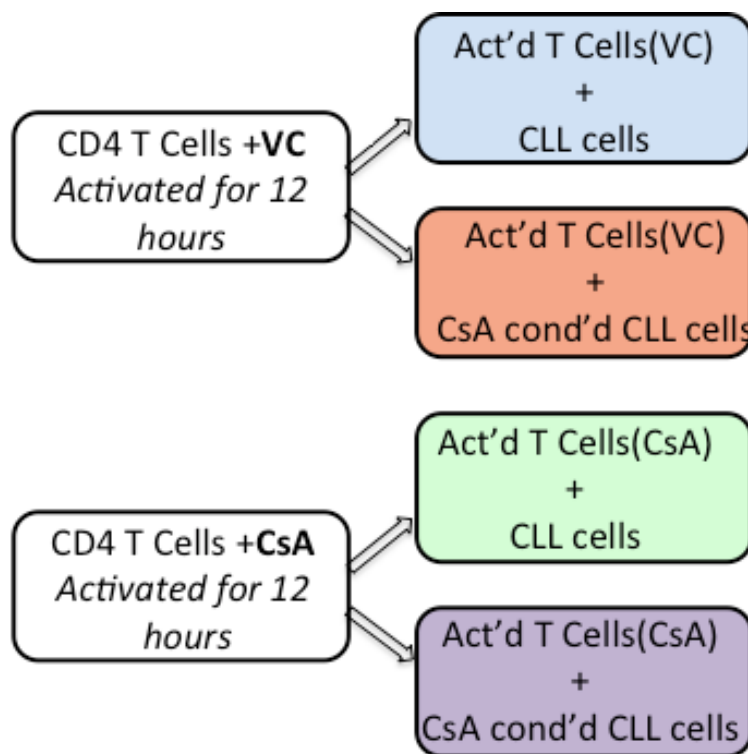


Figure 3.7 Co-Culture system to investigate the direct/indirect effect of CsA on CLL B cells

Autologous CD4+ T Cells were activated using anti-CD3/anti-CD28 for 12 hours in the presence of VC or CsA. After 12 hours, the activated cells were recombined with selected CLL B cells that had been pre-conditioned with CsA or VC and washed, at a ratio of 1:4.

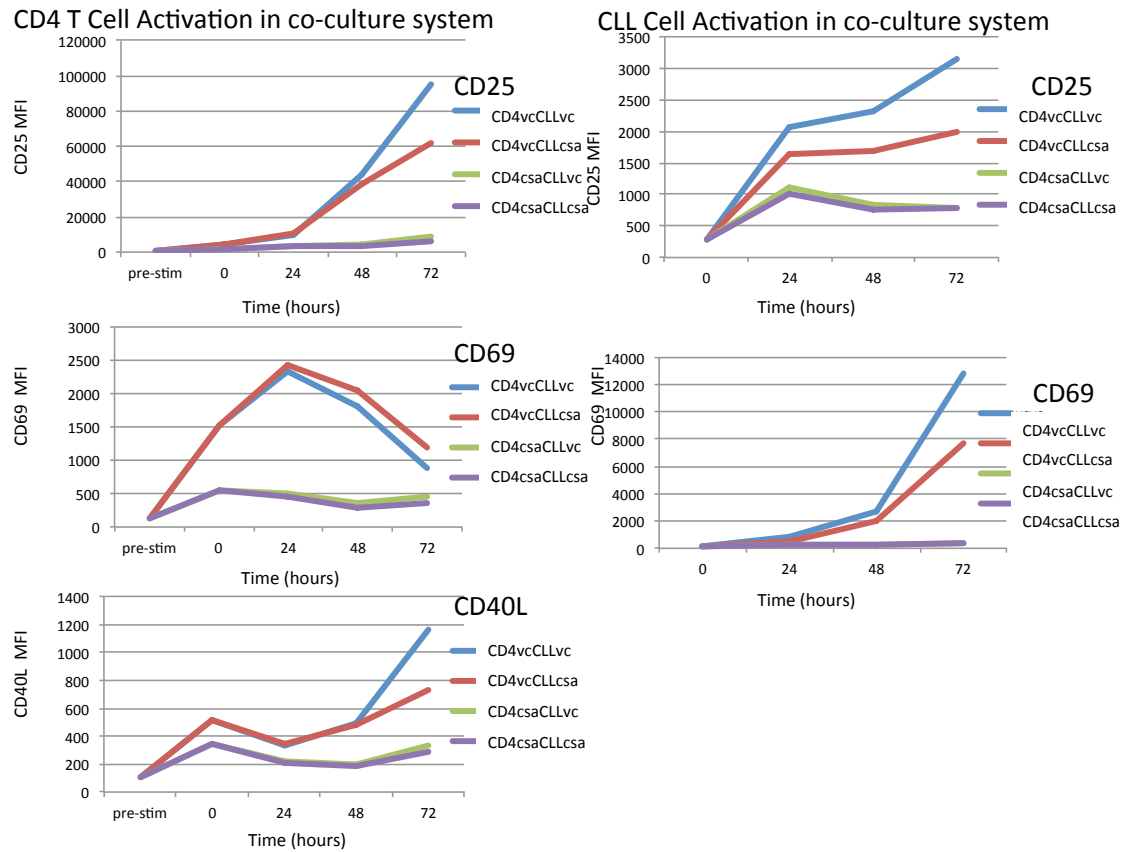


Figure 3.8 CsA has a major CD4 T cell dependent effect and a minor direct effect on activation of CLL cells in co-culture system

A representative plot of time-dependent activation of CD4 T Cells and CLL cells co-cultured as illustrated in Figure 3.7. The activation-status of CD4 T Cells and CLL B cells was assessed by flow cytometry daily for 3 days. Viable cells were gated on and the expression of surface markers is presented as Median Fluorescent Intensity (MFI).

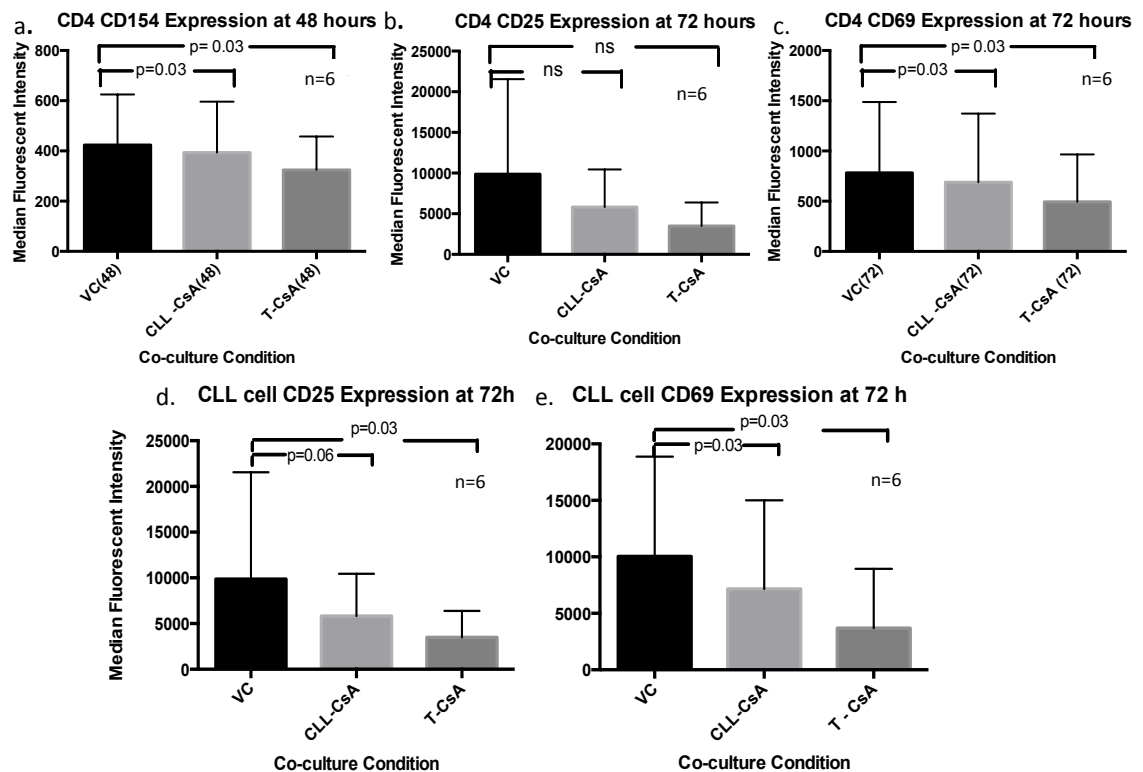


Figure 3.9 (a-e) CsA has a major T cell dependent effect and a minor direct effect on CLL cells activated with autologous CD4+ T Cells

(a-c) CD4 T cells were activated with anti-CD3/CD28 for 12 hours in the presence of CsA (T-CsA) or VC. They were then co-cultured with autologous CLL cells that had been pre-conditioned with CsA (CLL-CsA) or VC, for 72 hours. a-c illustrates activation status of CD4 T cells treated with CsA and VC and co-cultured with CLL cells for 72 hours. d-e illustrates activation status of CLL cells pre-conditioned with CsA/ VC and co-cultured with autologous CD4 T cells treated with CsA/ VC for 72 hours. Viable cells were gated on and the expression of surface markers is presented as Median Fluorescent Intensity (MFI). This figure summarises results of six experiments using six different patients.

3.3.7 Ciclosporin inhibits CD69 expression in healthy donor B cells and CLL cells activated through the B cell receptor

BCR signalling is considered to play a major role in CLL pathogenesis. CLL cells are variably responsive to BCR signalling; those that are unresponsive display features of anergy, with constitutive activation of NFAT and ERK, and are protected from apoptosis via expression of BCL2 and MCL1 (Muzio et al., 2008). Inhibition of NFAT signalling using the cell permeable VIVIT peptide has been shown to reduce metabolic activity of CLL cells in 'responders' and to reverse anergy and stimulate apoptosis in 'non-responders' (Apollonio et al., 2013; Le Roy et al., 2012) .

In view of the known effect of CsA on NFAT signalling we investigated the effect of CsA on healthy donor and CLL cells stimulated with agonistic anti-IgM.

Negatively selected healthy donor and CLL B cells were stimulated with goat F (ab')₂ anti human IgM for 24 hours in the presence and absence of CsA (200 ng/ml). Activation was flow-cytometrically assessed by measuring the proportion of cells expressing CD25 and CD69 after 24 hours. Figure 3.10 demonstrates that in anti-IgM-stimulated healthy donor B cells treated with CsA, CD69 expression was reduced from a mean of 66.5 % to a mean of 55.2 % (paired t-test, p=0.0018).

CLL cells were variably responsive to IgM stimulation, in our sample, 6/7 responded to sIgM ligation by upregulating CD25 and CD69 expression. Amongst the 'responders' CsA reduced CD69 expression from a mean of 70 % to a mean of 61.98 % (Wilcoxon matched pairs signed rank p = 0.03). There was no statistically significant effect of CsA on CD25 expression in healthy donor or CLL B cells activated through the BCR, suggesting that in contrast to CD69 expression, CD25 expression is regulated by a CsA insensitive mechanism.

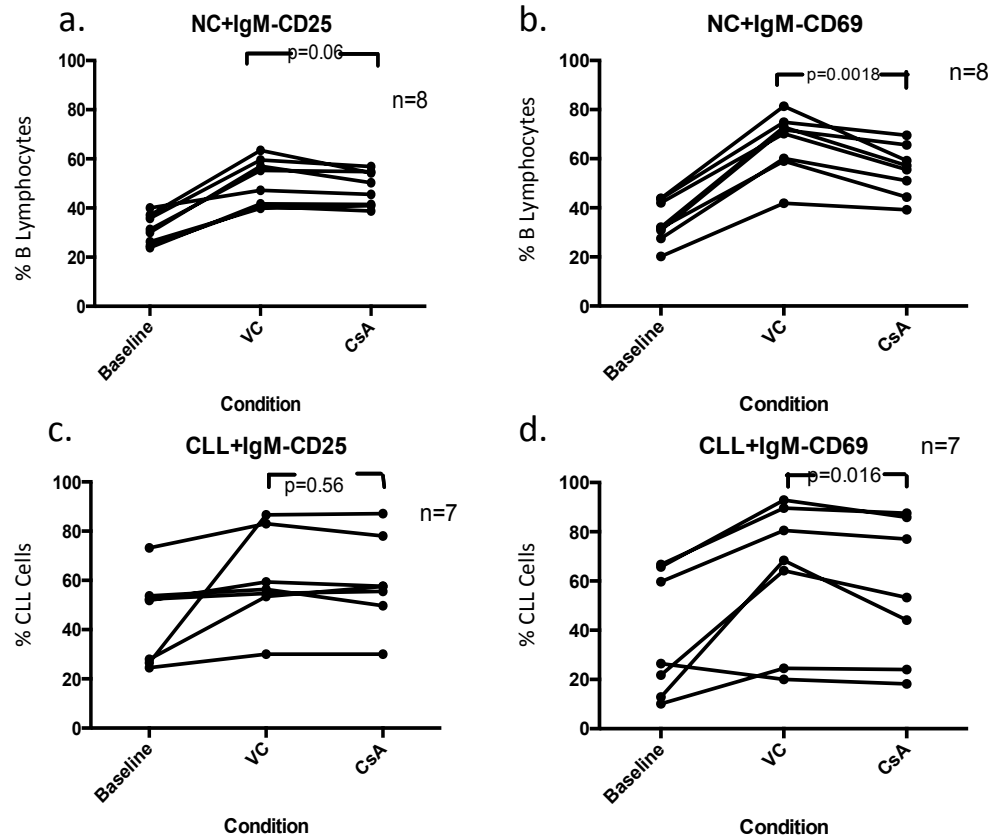


Figure 3.10 (a-d) Activation status of healthy donor B/ CLL cells stimulated in the presence of CsA or vehicle control

Selected healthy donor and CLL B cells were stimulated with IgM in the presence of CsA or VC. Expression of CD25 and CD69 was measured after 24 hours by flow cytometry. Viable cells co-expressing CD19 (healthy donor) or co-expressing CD5 and CD19 (CLL) were gated on (gates were set using fluorescence minus one controls). Percent parent denotes the percentage of B/ CLL cells expressing CD25 and CD69.

3.3.8 There is a trend towards reduced viability of CLL cells in the presence of ciclosporin

Having demonstrated that CsA inhibits both the activation of CLL cells stimulated through the BCR and CLL cells stimulated by autologous CD4 T cells, it was necessary to investigate the effect of CsA on CLL cell viability. NFAT and NF κ B signalling play a role in maintaining the viability of CLL cells and it is possible that CsA may disrupt these signalling pathways.

To investigate whether any effect of CsA on the viability of CLL cells was direct, or T cell dependent, cells were cultured as unselected PBMCs or as selected CLL cells. CLL cell selection was performed using immunomagnetic beads (Materials and Methods: Section 2.8) and the purity was confirmed to be $\geq 95\%$ CD5+/CD19+ by flow cytometry. Cells were cultured at a density of 3×10^6 for 96 hours in complete RPMI with CsA 200 ng/mL or VC. After 96 hours, viability was assessed by measuring non-viable cells with Annexin V and 7-Aminoactinomycin D (7-AAD). Annexin V is a phospholipid binding protein that binds to phosphatidylserine exposed on apoptotic cells and 7-AAD is a DNA intercalating dye that can only enter cells with disrupted cell membranes, indicating necrosis. An example of a flow-cytometry plot pre and post cell culture with and without CsA is shown Figure 3.11.

After 96 hours, there was a trend towards greater viability when CLL cells were cultured as PBMCs than when cultured as purified CLL cells (mean of differences = 10 %, paired t-test $p=0.059$), suggesting a protective effect of T cells. There was a trend towards reduced CLL cell viability in the presence of CsA in both CLL cells cultured as PBMCs (mean viability = 26.68 % vs 31.2 % (paired t-test, $p = 0.06$)) and as selected CLL cells (mean viability = 15.57 vs 21.08 % (paired t-test, $p = 0.07$)) as shown in Figure 3.12, suggesting that any effect on viability is a direct effect.

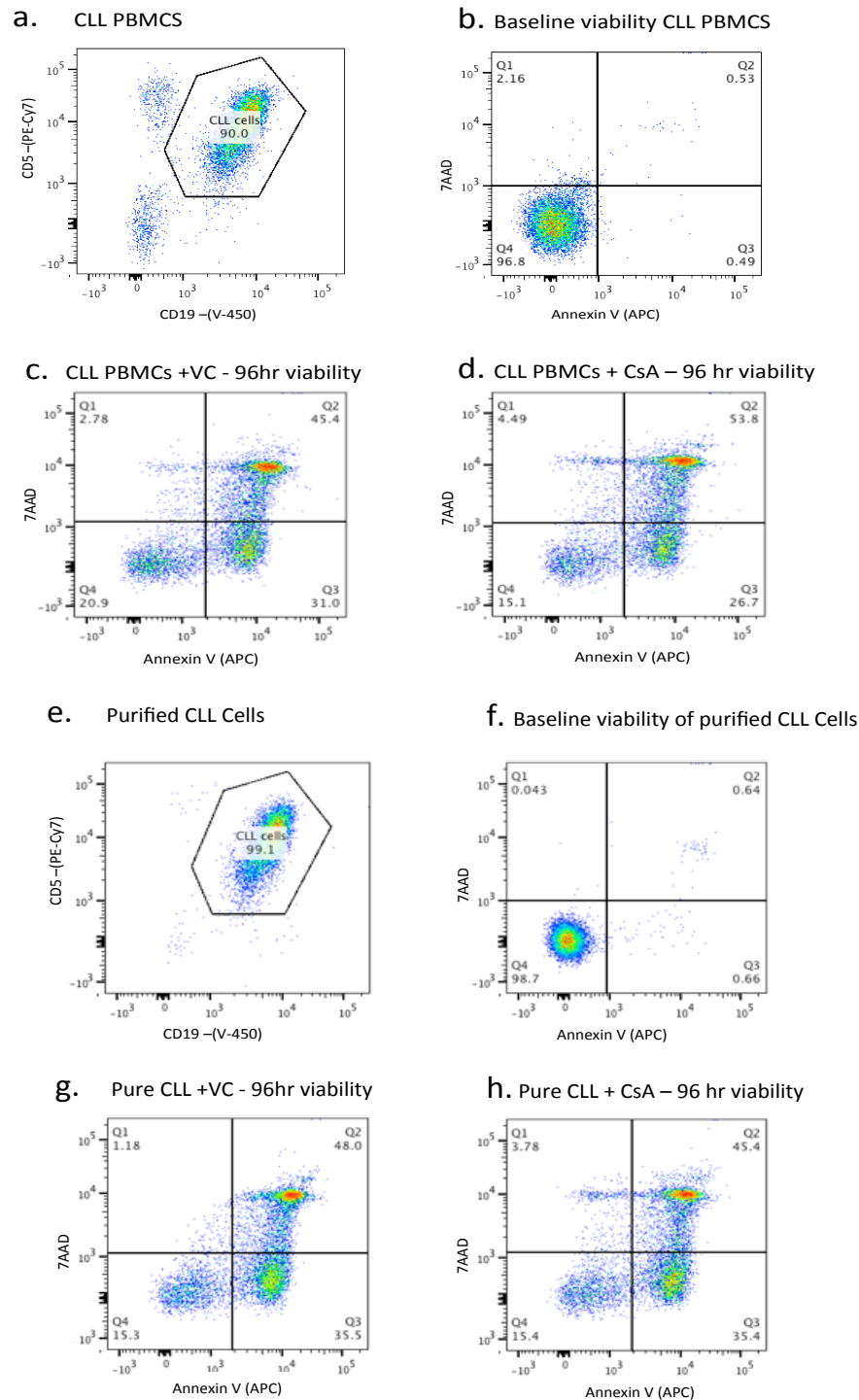


Figure 3.11 (a-h) Flow cytometry plots demonstrating viability of PBMCs pre-and post 96-hour culture with CsA or VC

(a-d) CLL cells were cultured as PBMCs with CsA 200 ng/mL or vehicle control (VC) for 96 hours. Viability was assessed flow- cytometrically by excluding cells staining positive for annexin V or 7-AAD. (e-h). Flow cytometry plots demonstrating viability of purified CLL cells pre-and post 96-hour culture with CsA or VC.

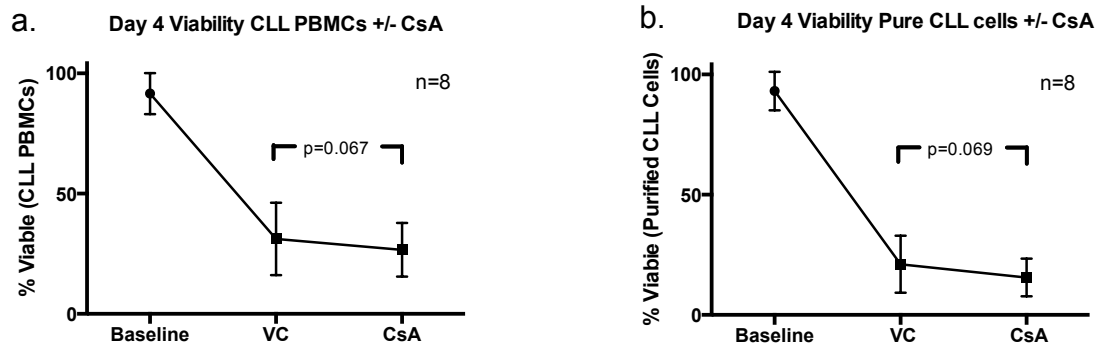


Figure 3.12 Viability of CLL cells cultured as PBMCs and as selected CLL cells in the presence of CsA

CLL cells were cultured as PBMCs or as selected CLL cells with and without CsA 200 ng/mL for 96 hours. Viability was assessed at baseline and after 96 hours by excluding cells staining positive for annexin V or 7-AAD.

3.3.9 Time-course of NF κ B and NFAT-C2 activation in normal CD4⁺ T cells stimulated in presence and absence of ciclosporin

The time-course of nuclear translocation of NF κ B-p65 and NFAT-C2 of CD4⁺ T cells was investigated by activating healthy donor PBMCs with PMA and Ionomycin. PMA plus Ionomycin was selected as the mechanism of as the combination has been shown to induce rapid and robust T cell activation. Ionomycin activates calcium/calmodulin-dependent signaling pathways, and induces the hydrolysis of phosphoinositides and the activation of protein kinase C (PKC) in human T cells. PMA is thought to act in synergy with Ionomycin to enhance activation of PKC.

Translocation was assessed at 10, 20, 30 and 60 minutes post stimulation. CD4⁺ T cells were gated on and the degree of nuclear translocation of NF κ B-p65 and NFAT-C2 was calculated using a similarity score between the nucleus (DAPI) and the transcription factor (FITC). The effect of CsA was investigated by simultaneously incubating the cells with VC or CsA for one hour pre-activation. Figure 3.13 demonstrates that nuclear NF κ B-p65 peaked at 30 minutes and nuclear NFAT-C2 peaked at 10-20 minutes post stimulation. In this example, nuclear translocation of NF κ B-p65 was reduced by 28.7 % at 30 minutes and nuclear translocation of NFAT-C2 was reduced by 57 % at 10 minutes in the presence of CsA. Therefore the time chosen for future experiments involving T cell activation was 30 minutes for NF κ B-p65 and 10 minutes for NFAT-C2.

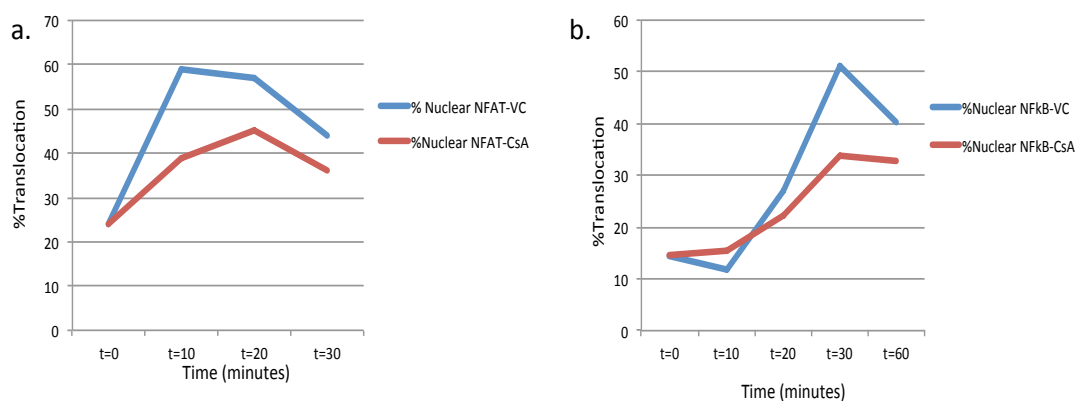


Figure 3.13 (a&b) Exemplary plots of time-course of nuclear translocation of NFAT-C2 and NF κ B-p65 in normal CD4⁺ T cells in presence of VC and CsA

PBMCs were activated with PMA and Ionomycin in the presence of VC or CsA. Percent nuclear translocation of NF κ B-p65 and NFAT-C2 at various time intervals is illustrated.

3.3.10 Time-course of NF κ B-p65 and NFAT-C2 activation in CLL cells stimulated by CD40L in presence and absence of ciclosporin

The time-course of nuclear translocation of NF κ B-p65 and NFAT-C2 in CLL cells in response to CD40L stimulation was investigated using CD40L transfected fibroblasts. Again, CD40L mediated activation was considered necessary to re-capitulate T cell activation of CLL cells. Figure 3.14 illustrates that nuclear translocation of NF κ B-p65 peaked at 3 hours post activation and was not inhibited in the presence of CsA. Nuclear translocation of NFAT-C2 peaked at 10-20 minutes and was transient, with nuclear translocation falling by 30 minutes. In the illustrated example, there was a reduction in NFAT-C2 translocation in the presence of CsA; it was reduced by 13 % at 10 minutes and by 43 % at 20 minutes.

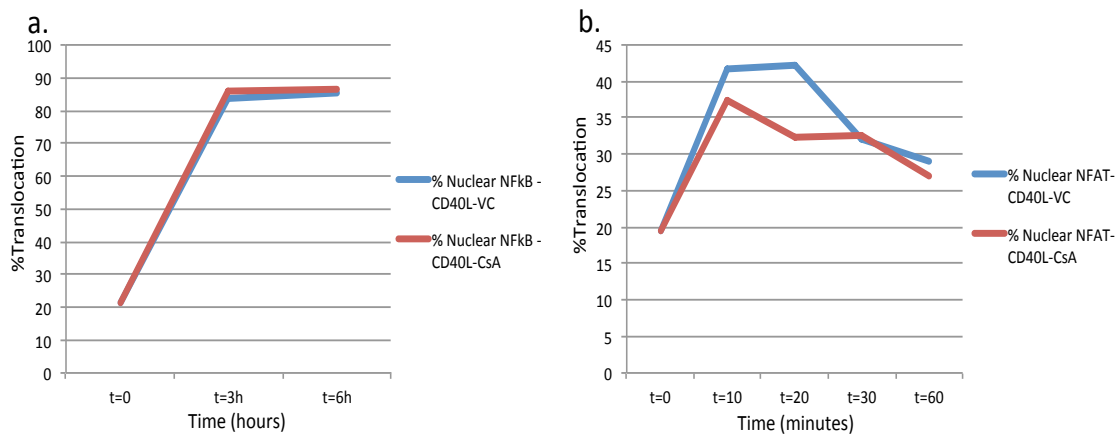


Figure 3.14 (a&b) Exemplary plot of time-course of nuclear translocation of NF κ B-p65 and NFAT-C2 in CLL cells in presence of VC and CsA

CLL PBMCs were co-cultured with CD40L transfected fibroblasts in the presence of VC or CsA for the times shown. CLL B cells were gated on and the degree of nuclear translocation of NF κ B-p65 and NFAT-C2 was calculated using a similarity score between the nucleus (DAPI) and the transcription factor (FITC).

3.3.11 Ciclosporin significantly inhibits NFκB-p65 and NFAT-C2 nuclear translocation in CLL CD4+ T Cells

Having established that NFκB-p65 nuclear translocation peaks at 30 minutes and NFAT-C2 translocation peaks at 10 minutes following PMA and Ionomycin stimulation of CD4 T cells, the effect of CsA on CLL CD4 T cells at these time-points was investigated. Translocation of NFκB-p65/NFAT-C2 was assessed in selected CD4 T cells from eight/nine CLL patients respectively. Figure 3.15 demonstrates that in the presence of CsA, NFκB-p65 translocation was reduced from a mean of 59.53 % to a mean of 35.63 % (paired t-test, $p < 0.0001$) and NFAT-C2 was reduced from a mean of 53 % to a mean of 36.5 % (paired t-test, $p = 0.005$).

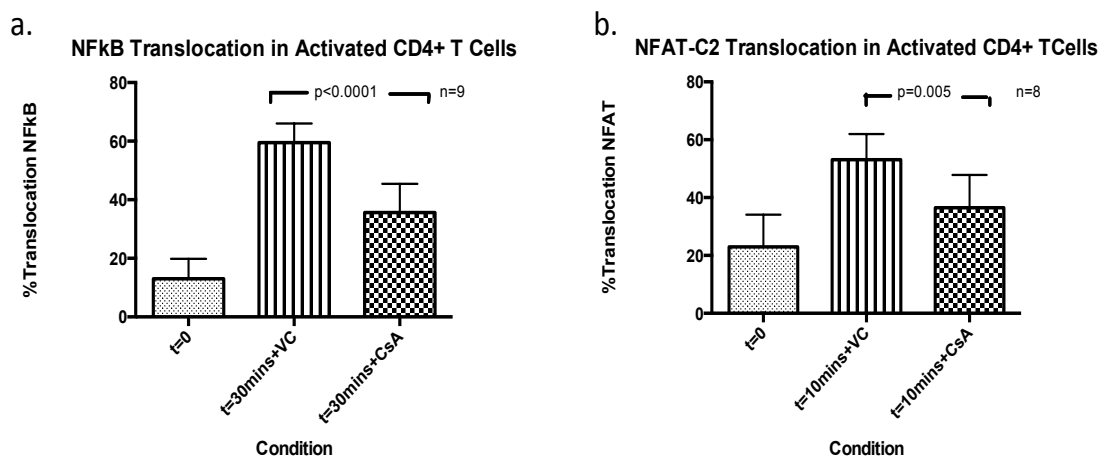


Figure 3.15 (a&b) CsA inhibits nuclear translocation of NF κ B-p65 and NFAT-C2 translocation in activated CLL CD4+ T cells

Selected CD4+ T cells were activated with PMA and Ionomycin for ten minutes following incubation with VC or CsA for one hour. CD4+ T cells were gated on and the degree of nuclear translocation of NFκB-p65 and NFAT-C2 was calculated using a similarity score between the nucleus (DAPI) and the transcription factor (FITC).

3.3.12 Effect of ciclosporin on NFκB-p65 and NFAT-C2 translocation in CLL cells

Having demonstrated that nuclear translocation of NFκB-p65 peaks at three hours and nuclear NFAT-C2 peaks at 10 minutes after activation of CLL B cells with CD40L transfected fibroblasts, CsA inhibition of translocation was measured at these time points. Figure 3.16 demonstrates that CsA does not significantly inhibit nuclear translocation of NFκB-p65 or NFAT-C2 in CD40L activated CLL cells at these time points. This is in agreement with the observation that CsA does not inhibit CD40L mediated activation of CLL cells as assessed by CD25 and CD69 expression. This observation is at odds with the well-documented effect of CsA on NFAT activation and potentially relates to the supra-physiological mechanism of CLL cell activation.

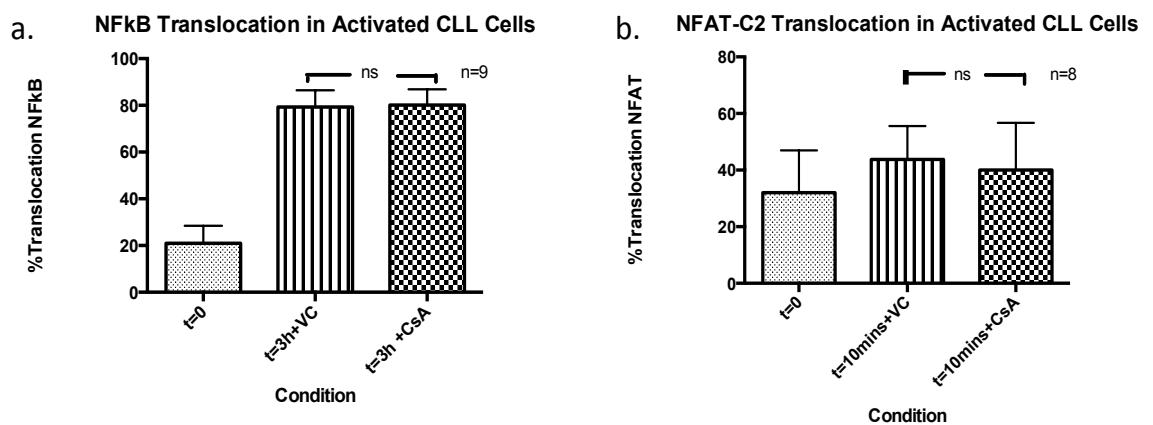


Figure 3.16 (a&b) Effect of CsA on NF κ B-p65 and NFAT-C2 translocation in CLL cells

Selected CLL cells were pre-incubated with VC or CsA for 60 minutes before being activated with CD40L transfected fibroblasts for the above times. CLL cells were gated on and the degree of nuclear translocation of NFκB-p65 and NFAT-C2 was calculated using a similarity score between the nucleus (DAPI) and the transcription factor (FITC)(n=8-9).

3.4 Discussion

The *in-vitro* assays presented here were designed to dissect the mechanism of action of CsA in patients with CLL; looking at the direct effect of CsA on CD4 T cells and CLL cells and on the interaction between them, as would be expected to occur in the CLL lymph-node.

We found that both healthy donor and CLL CD4 T cell activation, assessed by CD69 and IFN γ expression, was significantly inhibited in the presence of CsA (200 ng/ml). As it has previously been reported that IFN γ is a major mechanism by which CLL-specific Th1 cells drive the expression of CD38 by CLL cells (Bürgler et al., 2015), this finding suggests that CsA-treated CD4 T cells may have an impaired ability to activate CLL cells.

Previous investigations into the nature of CD4 T cell-mediated survival and activation of CLL cells in the lymph-node microenvironment have demonstrated that CD40L expression and IL21 secretion play a key role in this process (Ghia et al., 2002; Pascutti et al., 2013). As CsA has been demonstrated to inhibit CD40L expression and IL21 secretion in healthy T cells (Fuleihan et al., 1994; Kim et al., 2005), we went on to investigate the effect of CsA on CLL CD4 T cells. Importantly, we found that in the presence of CsA, there was marked inhibition of CD40L expression. In view of the fact that CD40L signalling has been shown to play a key role in CLL cell survival via the induction of *BCL-2* and *MCL-1* (Scielzo et al., 2011) and that blocking this axis using a monoclonal antibody to CD40L induces apoptosis (Furman et al., 2000), this would be predicted to reduce CLL cell survival. However, IL21 secretion was not reduced in the presence CsA, which goes against the known NFAT-dependent mechanism of transcription of the IL21 gene. One explanation for this observation is the high level of baseline fluorescence for IL21 and a blocking antibody may have been employed to establish a true-negative control.

We further interrogated the effect of CsA on healthy and CLL CD4 cells by measuring nuclear translocation of NFAT-C2 and NF κ B-p65 using image flow cytometry. We found that CsA had a significant effect on nuclear translocation of both NFAT-C2 and NF κ B-p65, reducing nuclear localisation by 30 % and 40 %, respectively. NFAT-C2 and NF κ B-p65 are key regulators of the transcription of IL2, IL4, IL6, IL21, TNF α , IFN γ and CD40L,

inhibition of which would be predicted to have a detrimental effect on the ability of CD4 T cells to activate CLL cells.

CD40L mediated activation of CLL cells by CD4 T cells in the lymph-node microenvironment is considered to play a key role in the survival and proliferation of CLL cells. We found that CD40L was a potent method of activating expression of CD25 and CD69 by CLL B cells and that this activation was not sensitive to the effect of CsA. Furthermore, NF κ B-p65 and NFAT-C2 translocation was induced in CD40L activated CLL cells, and was insensitive to CsA. This finding is in-keeping with *in-vitro* studies that have demonstrated that CD40L-induced proliferation of healthy B lymphocytes is resistant to the effect of CsA (Wortis et al., 1995). However, it is at odds with the finding that in B lymphocytes, NFAT signalling induced by CD40L + IL4 is sensitive to the effect of CsA (Choi et al., 1994). IL4 signalling is mediated by STAT6 phosphorylation and this mechanism of activation has recently been shown to be upregulated in CLL cells compared to healthy B lymphocytes; however sensitivity of IL4 signalling to CsA has not been investigated (Bhattacharya et al., 2015).

Investigation into the effect of CsA on the interaction between activated CD4 T cells and CLL cells in the co-culture system was designed to re-capitulate the CLL: T cell interactions in the lymph-node compartment. Pascutti et al have demonstrated that compared to isolated CD40L mediated activation, CD4 T cells induce a different gene expression profile and that chemokine signalling molecules account for this difference (Pascutti et al., 2013). Co-culture experiments demonstrated that CsA had a major inhibitory effect on CD4 T cells, which impaired their ability to activate CLL cells. In addition to the indirect, CD4 T cell dependent effect of CsA on CLL cells, the drug had an additional direct effect on CLL cells, as evidenced by reduced expression of CD69 by CsA pre-conditioned CLL cells co-cultured with untreated activated CD4 T cells. Furthermore, CsA-treated CLL cells provided less reciprocal CD4 T cell activation, further emphasising the role of bi-directional signalling between CLL and CD4 T cells.

The effect of CsA on BCR mediated activation was an important line of investigation in view of the profound effect that ibrutinib and idelalisib that inhibit BCR signalling via targeted inhibition of BTK and PI3K δ respectively (Herman et al., 2010) (Herman et al., 2011). CsA is known to inhibit NFAT that plays a key role in BCR mediated signalling in

CLL cells and furthermore, inhibition of NFAT signalling using the VIVIT peptide has been shown to reduce metabolic activity in both anergic and signalling-competent CLL cells and to reduce tumour volume in the MEC-1 mouse model of CLL (Apollonio et al., 2013; Le Roy et al., 2012). Secondly, NF κ B-p65 plays an important role in BCR signalling and the survival and proliferation of CLL cells, and has been shown to be sensitive to CsA in T cells.

Healthy donor and CLL cells activated through the BCR showed reduced CD69 expression on the presence of CsA, consistent with reports that CsA inhibits IgM induced NFAT activation and B cell proliferation (Venkataraman et al., 1994) (Wortis et al., 1995). However, CD25 expression was not suppressed in either healthy or CLL B cells in the presence of CsA, suggesting that a CsA-insensitive pathway in CLL cells regulates expression of this molecule. Owing to technical difficulties with the ImageStream™, the effect of CsA on NFAT-C2 and NF κ B-p65 activation in CLL cells activated through the BCR was not investigated in this study but remains an important line of investigation.

Finally, in view of the effect of CsA on BCR signalling and in view of the effect of CsA on CLL T cells that protect CLL cells against apoptosis, the effect of CsA on the viability of CLL cells cultured in isolation or as PBMCs was investigated. There was a trend towards increased viability when CLL cells were cultured as PBMCs and there was a similar trend towards reduced viability in the presence of CsA regardless of the presence of T cells. Any effect of CsA on CLL viability would therefore appear to be an independent of T cells.

In summary, CsA has a complex and heterogeneous effect on CLL: CD4 T cell interaction. Although CsA primarily reduced CD4 T cell mediated activation of CLL cells through inhibition of IFN γ and CD40L expression, there was a direct effect of CsA on CLL cells, notably reducing the ability of CLL cells to respond to CD4 T cell-mediated activation. The fact that CD4 T cell-induced activation of CLL cells was sensitive to CsA whilst CD40L mediated activation was resistant to CsA may be due to both qualitative and quantitative differences in the mode of activation including cytokine signalling.

There was a direct effect of CsA on CLL cells activated through the BCR in signalling competent cases, suggesting that CsA has a further, CD4 T cell-independent effect in these cases. Additionally, the trend towards reduced viability seen in CsA-treated CLL cells is consistent with the effect of the cell permeable VIVIT peptide that inhibits NFAT translocation and reduces viability in both anergic and signalling-competent cases (Apollonio et al., 2013; Le Roy et al., 2012).

The *in-vitro* studies provide evidence of a two-pronged anti-leukaemia mechanism of action of CsA, involving both CD4 T cell-dependent and direct mechanisms of action that explains why a proportion of patients with CLL treated with CsA experience tumour-regression. However, the fact that there was evidence of CsA-insensitive CLL cell activation in cells activated through the BCR emphasises the complexity of signalling that is likely to be further complicated by interactions between CLL cells, endothelial cells and nurse-like cells in the lymph-node compartment.

Based on the positive findings of the effect of CsA *in-vitro*, a clinical trial was performed to investigate the *in-vivo* effect of CsA in patients with poor risk disease who do not meet conventional criteria for treatment and in whom investigation of novel treatment strategies is warranted. The *in-vitro* studies of CsA in CLL were applied to investigate the *in-vivo* effect of CsA in patients recruited to the clinical trial.

Chapter 4 . Manipulation of the Tumour Microenvironment: An *In-vivo* Study of Ciclosporin in Chronic Lymphocytic Leukaemia

4.1 Introduction

4.1.1 Background and Rationale

CLL is a highly heterogeneous disease with clinical outcomes reflecting significant differences in disease biology. Determination of the biological characteristics of the disease, for example *IgV_H* gene mutational status and expression of CD38 and CD49d, are useful in predicting time to first treatment and overall survival and therefore provide relevant information to both the patient and clinician at the time of diagnosis (Hamblin et al., 1999) (Bulian et al., 2014; Damle et al., 1999; Hamblin et al., 2002). Median survival was reported to be 109 months in patients with either unmutated *IgV_H* genes (*IgV_H*-UM), or CD38 positive expression (>30 %) and was 293 months in patients who lacked either of these inferior prognostic markers. Although there was a correlation between CD38 expression and unmutated *IgV_H* genes, there was discordant expression in 28 % patients, in whom progression free survival was intermediate (Hamblin et al., 2002).

Investigators have since established that a threshold of 7 % CD38 expression is a more stringent discriminator of prognosis; with median overall survival estimated to be 114 months in patients with <7 % CD38 compared to 79 months in those with >7 % CD38 expression (Kröber et al., 2002; Thornton et al., 2004).

Identification of poor-risk characteristics does not, however, influence the decision to commence treatment and can therefore be psychologically damaging to patients. Treatment is reserved for patients with symptomatic, progressive disease based on evidence that early intervention with Chlorambucil does not improve survival (Dighiero et al., 1998). The cohort of patients with poor risk characteristics, destined to have a short time to first treatment represents an area of unmet medical need. Identification of a strategy that can be applied to slow the rate of disease progression before patients meet the conventional criteria to start treatment (Hallek et al., 2008) is highly

desirable. A treatment strategy applied in this setting should be non-toxic, effective and should inhibit rather than contribute to the genetic evolution of the tumour.

For many years, CLL was considered to be a disease of failed apoptosis (Kitada et al., 1998) but a landmark study using *in-vivo* deuterium labelling in CLL identified that in patients with progressive disease, proliferation rates can reach 2 % per day (Messmer et al., 2005). Although neither CD38 expression or *IgV_H* mutational status identified patients with high proliferation rates, it was subsequently reported that within a patient, CD38+ CLL cells proliferated at twice the rate of CD38- CLL cells (Calissano et al., 2009). This study therefore provided a key link between CLL proliferation and inferior prognosis in patients with CD38+ CLL.

Proliferation of CLL cells occurs in secondary lymphoid tissue, where there is evidence of active signalling through the B cell receptor (BCR) (Herishanu et al., 2011). However, the survival and proliferation of tumour cells is also thought to depend on signalling from components of the microenvironment including activated CD4 T cells, stromal cells, endothelial cells and nurse-like cells (Buggins et al., 2010; J. A. Burger et al., 2000; Panayiotidis et al., 1996). As noted in chapter 1, our group and others have investigated the role of activated CD4 T cells in the lymph-node microenvironment (Ghia et al., 2002; Patten, Buggins, Richards, Wotherspoon, Salisbury, Mufti, Hamblin, & Devereux, 2008b; Willimott et al., 2007) (Bagnara et al., 2011) (Pascutti et al., 2013). In light of the evidence for the role of CD4 T cells in promoting CLL survival and proliferation, we hypothesised that CD4 T cell suppression may inhibit CLL cell activation and proliferation. Furthermore, since signals from CD4+ T-cells induce the DNA modifying enzyme activation induced cytidine deaminase (AID), which has previously been implicated in the induction of mutations in CLL, targeting these cells might also reduce the genomic evolution of the disease. Manipulation of the tumour microenvironment in this way could be a relatively non-toxic mechanism of inhibiting disease progression in patients destined to have a poor outcome.

4.1.2 Ciclosporin

There are a number of agents that are applied in clinical medicine that have an anti-T cell effect; these include polyclonal antibodies e.g. antithymocyte globulin (ATG), monoclonal antibodies e.g. muromonab-CD3, drugs that inhibit the immunophilins e.g. ciclosporin (CsA), tacrolimus and sirolimus, and small biological agents including fingolimod. CsA was selected as the investigational agent to suppress T cell activation as most clinical experience has been gained with this agent, including in the context of CLL, where it has been shown to lead to tumour regression in approximately 20 % cases treated for autoimmune complications (Cortes et al., 2001).

CsA is a fungal derived polypeptide that was discovered to have anti-T cell activity in 1976 (Borel et al., 1976). CsA inhibits T cell activation by forming a complex with cyclophilin; the complex binds to calcineurin and blocks its phosphatase activity, which is required for the nuclear translocation of cNFAT. This, in turn, inhibits the nuclear transcription of IL2, IL4 and CD40L required for the growth and differentiation of T cells. CsA was first applied to the field of organ transplantation in the early 1980s where it now represents the standard of care in preventing graft rejection. CsA was subsequently applied to autoimmune conditions where it has been shown to be a relatively safe and effective method of treating a variety of conditions including rheumatoid arthritis, psoriasis and bullous pemphigoid. In the setting of CLL, CsA has been used to treat autoimmune haemolytic anaemia (AIHA) and pure red cell aplasia (PRCA) and has been reported to have anti-tumour efficacy (Cortes et al., 2001).

CsA is an orally available drug with minimal metabolism in the gastrointestinal tract. The drug is predominantly absorbed from the small bowel after which it enters cells by diffusion. Metabolism takes place in the liver by the cytochrome CYP3A system. The recommended dose in autoimmune conditions is 5 mg/kg/day, targeting a trough concentration of 150-200 ng/ml to minimize renal toxicity (Moyer, Post, Sterioff, & Anderson, 1988). Toxicity related to CsA therapy can be separated into those related to the chemical effect of the drug and those related to immune suppression. Toxicities are listed in Table 4.1

Table 4.1 Ciclosporin Toxicities

System	Toxicity
Renal	Hypertension, haemolytic uraemic syndrome
Liver	Hepatic dysfunction
Gastrointestinal	Gastrointestinal disturbance, pancreatitis
Haematological	Anaemia, thrombocytopenia, microangiopathic haemolytic anaemia
Neurological	Tremor, headache, paraesthesia, encephalopathy, demyelination, motor polyneuropathy, benign intracranial hypertension
Endocrine	Menstrual disturbance, hyperglycaemia
Musculoskeletal	Myalgia
Immunological	CMV reactivation, EBV reactivation
Electrolyte disturbance	Hyperkalaemia, hypomagnesaemia, hyperuricaemia
Other	Anorexia, gingival hyperplasia, fatigue, hyperlipidaemia, hypertrichosis

Before embarking on the clinical trial, a series of assays was developed to investigate the *in-vitro* effect of CsA and to subsequently be applied to the clinical trial to investigate the *in-vivo* effect of CsA in patients with CLL.

The *in-vitro* studies, discussed in depth in Chapter 3, demonstrated that CsA inhibited nuclear translocation of NFAT-C2 and NFκB-p65 in activated CLL CD4 T cells. Furthermore, there was reduced expression of IFNγ and CD40L in CD4 T cells activated in the presence of CsA; IFNγ and CD40L are thought to play a key role in T-cell activation of CLL cells in the lymph-node compartment. In the co-culture system designed to replicate CD4 T cell: CLL interaction in the lymph-node compartment, treatment of CD4 T cells with CsA appeared to indirectly reduce CLL cell activation.

In addition to the CD4 T cell-dependent effect of the drug, CsA had a direct effect on CLL cells. Not only did CsA inhibit the ability of CLL cells to respond to activation by autologous CD4 T cells, it inhibited activation of CLL cells stimulated through the BCR. Finally, there was a trend towards reduced viability of CLL cells in the presence of CsA, consistent with the fact that CsA inhibits NFAT and NFκB-p65 which are known to play a role in maintaining viability. In summary, the *in-vitro* studies demonstrated a major CD4 T cell dependent effect and a smaller direct effect of CsA on CLL cells. These studies concluded that CsA has *in-vitro* activity in CLL and provided an important foundation for developing a clinical study.

4.1.3 Study Design and Endpoints

The clinical trial was designed to investigate the effect of CsA on proliferation of CLL cells in ten patients with early stage, adverse risk (defined by CD38 expression > 7 %) disease. The 'Ciclosporin in Early Stage, Adverse Risk Chronic Lymphocytic Leukaemia' Trial (CyCLLe) was a Phase 2 Study set-up and co-ordinated by Bloodwise Trials Acceleration Program and sponsored by the University of Birmingham.

The primary end-point of the study was change in proliferation after four weeks of CsA treatment. As proliferation takes place in the lymph-node compartment, a 'whole-body method' of measuring proliferation was necessary. Deuterium labelling, which has previously been applied to measure the proliferation of CLL cells (Defoiche et al., 2008; Messmer et al., 2005) was considered as the best method for measuring proliferation.

Deuterium is a stable, non-radioactive isotope of hydrogen that can be delivered orally as either deuterated water or deuterated glucose. *In-vivo* measurement of proliferation using deuterated glucose was first described by Prof. D. Macallan and has been used to study highly proliferative cell populations, particularly lymphocyte populations (Macallan et al., 1998). The advantage of using deuterated glucose is that the body glucose pool is small (approximately 15g) and that it turns-over rapidly, such that a well-defined pulse of cells can be labelled in a single day and tracked over time. In comparison, the body water pool is large, labelling requires ingestion of deuterated

water daily for 84 days and there is slow turnover, leading ongoing uptake of label during the wash-out phase.

Deuterated glucose labelling is performed in semi-fasting state. Enrichment of the plasma-glucose compartment is rapidly achieved following a priming dose of deuterated glucose. Enrichment is sustained for a ten-hour period by administration of aliquots of labelled glucose every 30 minutes and by limiting oral intake of un-labelled glucose/ carbohydrate. After entering cells, glucose-6-phosphate is converted non-oxidatively to ribose-5-phosphate, then further phosphorylated to phosphoribose pyrophosphate (PRPP), which is the basis for *de novo* synthesis of both purine and pyrimidine nucleotides.

Following cessation of labelling, the cell population of interest can be serially sampled over time to assess rates of appearance/ disappearance and proliferation. The cells are isolated, purified and DNA is extracted. DNA is hydrolysed, derivatised (using pentafluoro tri-acetate (PTFA)) and the degree of deuterium enrichment measured using gas chromatography mass-spectrometry (GC-MS). In this study, aliquots of 100,000 cells were transferred to Prof Macallan's lab where Dr. Zhang performed DNA extraction, hydrolysis, derivatisation and GC-MS. Deuterium enrichment of adenosine is preferentially measured as purines are synthesized via the *de novo* nucleotide pathway rather than by the nucleoside salvage pathway (Macallan et al., 2009).

As proliferation occurs in the lymph-node compartment, it was assumed that cells are initially labelled there and then traffic to the peripheral blood. Therefore, secondary trial endpoints included rates of appearance of labelled cells from the lymph-node compartment into the peripheral blood, followed by their later disappearance. Measurement of release rates of labelled cells into the peripheral blood was considered to be an important line of investigation in view of the *in-vitro* observation that CsA inhibits expression of CD69 by CLL cells, which would promote their egress into the peripheral circulation. CD69 has been shown to promote the retention of lymphocytes in lymphoid organs by acting downstream of INF α/β and to down-modulate sphingosine 1-phosphate receptor-1 (S1P1) expression that is required for lymphocyte egress (Shiow et al., 2006). Disappearance rates of CLL cells were

measured in view of the trend towards increased apoptosis of CLL cells in the presence of CsA.

The clinical response to CsA therapy at three months post treatment and the toxicity of therapy were additionally collected as secondary outcome measures.

Translational studies developed during the *in-vitro* phase of this investigation were applied to further dissect the mechanism of action of CsA *in-vivo*. Although it is recognised that CLL: CD4 T cell interaction and CLL proliferation takes place within the lymph-node, all studies were performed on peripheral blood CLL cells. Studies on lymph-node derived cells were not feasible within the clinical trial. Translational studies included flow cytometry assessment of the activation status of CD4 T cells and CLL cells, image-flow cytometry assessment of NFAT-C2 and NFκB-p65 translocation and the gene expression profile of CD4+ T cell and CLL cells pre and during CsA treatment.

It has previously been demonstrated that the T cell compartment in CLL is skewed towards an effector memory phenotype that correlates with increased expression of the exhaustion markers CD244, CD160, PD-1 and Blimp1, consistent with an antigen experienced phenotype (Riches et al., 2013). We therefore assessed whether CsA treatment impacted on this phenotype by assessment of T cell subsets and expression of PD-1 pre-and after four weeks' *in-vivo* exposure to CsA.

4.2 Materials and Methods

4.2.1 Trial Set-Up and Management

The trial was designed by Prof Stephen Devereux (Chief Investigator) in collaboration with co-investigators Prof Macallan, Prof Fegan, Prof Pepper, Dr Stocken and Dr Cuthill. The trial was funded by and performed with support from the Bloodwise Trials Acceleration Program (TAP) and co-ordinated by Sonia Fox (Cancer Research Clinical Trials Unit, Birmingham). The trial was granted ethical approval (REC:12/EE/0485) and local approval from the Department of Research and Development. The trial was registered on the EudraCT database (2012-002795-13). The trial was opened at two centres; King's College Hospital and University Hospital Wales, Cardiff (UHW). The trial was compliant with the principles of Good Clinical Practice and all laboratory work complied with the principles of Good Laboratory Practice.

4.2.2 Trial Overview

CyCLLe was a Phase II Single Arm study with the objective of investigating the effect of CsA on tumour kinetics in CLL (for Protocol, see Appendix 1). Outcome measures are listed below:-

Primary Outcome Measures:

- Change in proliferation rate after four weeks of CsA therapy, measured by deuterated glucose incorporation

Secondary Outcome Measures:

- Toxicity of CsA in patients with CLL
- Spontaneous *intra*-patient variation in proliferation, release and loss of CLL cells from the circulation
- Time to maximum release of labelled CLL cells from the circulation with CsA therapy
- Complete response rate
- Overall response rate

Translational Outcome Measures:

- Effect of CsA on phenotype of CLL and T cells
- Effect of CsA on transcription factors NFκB-p65 and NFAT
- Gene expression profile of selected CLL and CD4 T cells

4.2.3 Eligibility Criteria

Inclusion Criteria

- Binet Stage A or B CLL not requiring treatment by conventional criteria
- Age ≥ 18
- ECOG performance status ≤ 2
- Life expectancy > 12 months
- No therapy for CLL in previous 3 months (including glucocorticoids)
- CD38+ $\geq 7\%$
- Normal renal function
- Normal liver function
- Negative serology for Hepatitis C, HIV and active Hepatitis B infection
- Valid informed consent

Exclusion Criteria

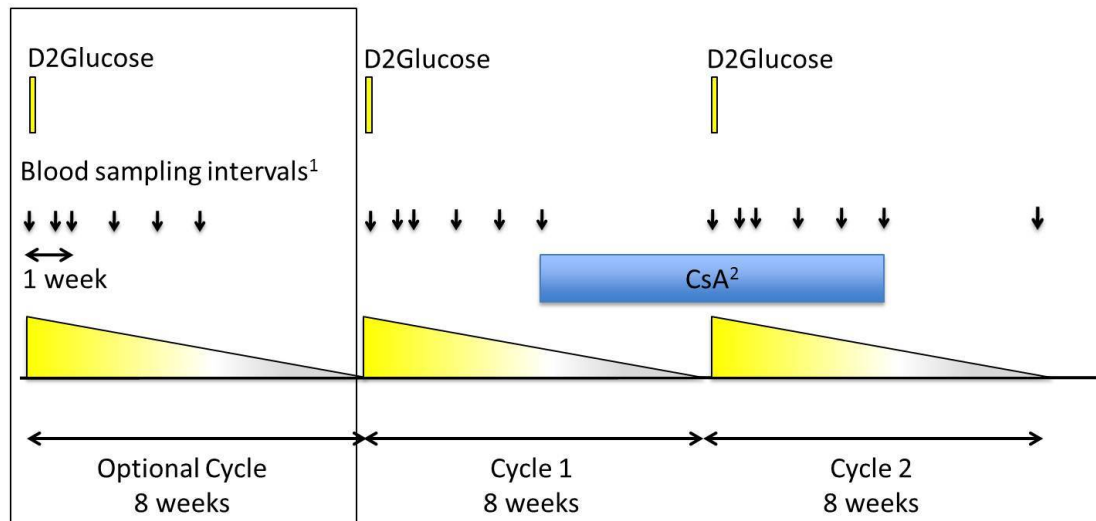
- Active infection
- Active autoimmune disease requiring therapy
- Diabetes Mellitus
- Previous myocardial infarction or clinically significant cardiac dysrhythmia
- Uncontrolled hypertension
- Taking medication known to have serious interaction with CsA where interaction cannot be managed by adjusting CsA dose
- Fludarabine refractory disease
- Previous bone marrow transplant

- History of prior malignancy, with the exception of certain malignancies treated with curative intent and with no evidence of active disease for more than three years
- Pregnant and lactating patients (patients of childbearing potential should have a negative pregnancy test)
- Patients and partners of childbearing potential not willing to use effective contraception during and for three months after the study

4.2.4 Outline of the CyCLLe study

The study was initially designed to include three labelling days, enabling assessment of proliferation on two occasions to investigate intra-patient variation in proliferation before the commencement of CsA. Due to the difficulty in recruiting patients, the protocol was amended to make the first cycle optional, followed by two compulsory cycles, as illustrated in Figure 4.1. Labelling with deuterated glucose was performed on D0 and compulsory blood tests were drawn on days 4, 7, 14, 21, 28 and 56- post labelling. Assessment of release rate of labelled cells into the peripheral blood was an optional extra and involved blood tests on days 1, 2, 3 post labelling. CsA was administered for 56 days and there was an option to continue treatment for up to six months in responding patients.

Outline of the CyCLLe study



¹ Not including optional samples to measure release rate (days 1-3 of each cycle)

² Responding patients will have the option of continuing CsA for a total of 6 months or until progression or toxicity supervene

Figure 4.1 Outline of CyCLLe

The study included two compulsory cycles and one optional cycle. CsA therapy was commenced on D28 of the second cycle. Bloods for deuterium assessment were drawn at baseline, on day 4, weekly for four weeks and then after 8 weeks.

4.2.5 Trial Recruitment

Patients were provided with a Patient Information Sheet and were given adequate time to make a decision regarding participation. After the patient agreed to participate, they signed an informed consent form, following which they were screened for eligibility. Full screening evaluation included medical history, physical examination, blood tests and ECG. Once the patient was confirmed to be eligible for trial entry, they were registered via the Cancer Research UK Clinical Trials Unit. Dr Cuthill approached all patients and arranged all consent and screening visits. In total, fourteen patients were approached, seven consented to screening and five were eligible for trial entry. Four patients were recruited from King's College Hospital and one from UHW.

4.2.6 Deuterium Labelling

Deuterated Glucose (Cambridge Isotope Laboratories), 60 g, was reconstituted with 200ml water and left to dissolve over-night. Patients were advised to eat a light breakfast and to attend early on the labelling day (Day 0). On arrival, patients underwent a physical examination for fitness to proceed. Baseline blood was drawn (1ml EDTA) and frozen at -20 °C. Three drops of blood were placed on a filter paper and allowed to air-dry. 36 ml D2-glucose was given at baseline and 10 ml at thirty-minute intervals for ten hours. Finger-prick blood tests were performed at one, three, five eight and ten hours, with blood spots allowed to air-dry on filter papers before storing at -20 °C and shipping to St George's Hospital. During the day, the patient was allowed to eat three half-portions of low carbohydrate, low-sugar food, specifically at three hours, five hours thirty minutes and eight hours thirty minutes. Labelling was repeated after eight weeks. Dr. Cuthill arranged and co-ordinated all labelling appointments and King's College Hospital and trained staff at Cardiff University Hospital.

4.2.7 Blood Sampling

5ml EDTA blood samples were drawn on days 4, 7, 14, 21 and 28. If the patient consented to the optional release-rates study, 5 ml EDTA blood samples were additionally drawn on days 1, 2, 3 post labelling. Blood sampling at KCH was performed by Dr. Cuthill with the occasional help of Clinical Trials nurses.

4.2.8 CLL cell isolation – for assessment of deuterium incorporation

CLL cells were positively selected using CD19 microbeads (Miltenyi) as per the manufacturer's instructions. Positive selection was used as this provided an efficient yield of CD19 cells with a purity of >95 %. 10^7 PBMCs were pelleted at 300 *g* for 10 minutes and the supernatant removed by aspiration. Cells were resuspended in 80 µL running buffer (containing PBS, 0.5 % (w/v) bovine serum albumin and 2nM EDTA, pH

7.2) and 20 μ L CD19 microbeads. Cells were incubated at 4°C for 15 minutes to enable labeling, following which, cells were washed with 1 mL running buffer and re-suspended in 500 μ L running buffer for selection using the Automacs™ cell separator. Selected CD19 cells were aliquoted at 1×10^6 cells into Eppendorf microtubes and pelleted at 12000 *g* for 3 minutes. The supernatant was aspirated and cell pellets stored at -80°C until they were shipped to St George's Hospital for gas chromatography mass spectrometry assessment of deuterium incorporation in Prof Macallan's laboratory. CLL purity was measured at least once for each patient and when a new positive selection kit was introduced using flow cytometry; an example of the purity check is illustrated in Figure 4.2

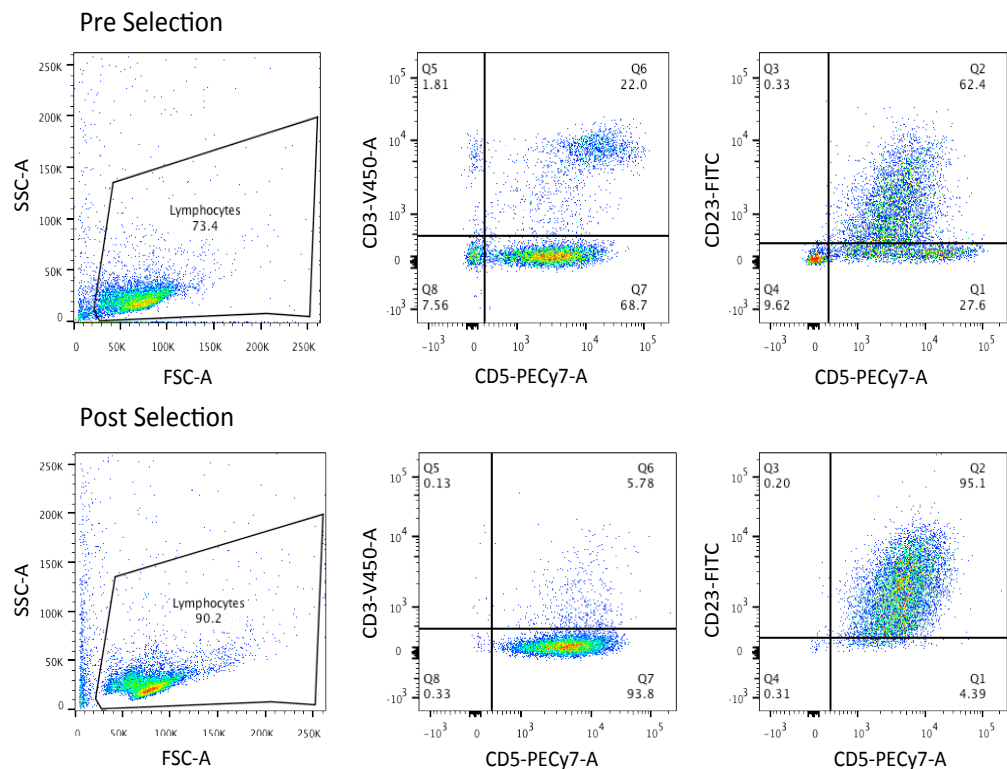


Figure 4.2 Example of CLL purity check by flow cytometry

The purity of CLL cells was measured pre and post positive-selection. Purity was assessed by flow cytometry. Purity >95% was considered acceptable.

4.2.9 Assessment of deuterium incorporation

Aliquots of purified cells and filter papers (whole blood glucose) were transferred to Dr Yan Zhang (Prof D. Macallan, Institute of Infection and Immunity, St George's Hospital, University of London) where analysis of deuterium enrichment was performed. Dr. Yan Zhang performed all sample preparation and deuterium enrichment analysis; Prof Macallan reviewed and modeled data. Calculation of whole blood glucose deuterium enrichment over the ten-hour labelling period was necessary for calculation of the fraction of new DNA. An example of whole blood glucose enrichment from a subject labelled in the CyCLLe study is illustrated in Figure 4.3.

- a. Assessment of blood glucose enrichment:
 - i. Glucose extracted from filter papers.
 - ii. Glucose derivatised and transferred to vials.
 - iii. Gas-chromatography mass spectrometry (GC-MS) analysis using selective monitoring for ions of 328 and 330.
 - iv. Enrichment determined from ratios of ions $M+2/[M+0+M+2]$, calibrating against standard glucose samples of known enrichment.
 - v. Area under curve for glucose enrichment vs time calculated.

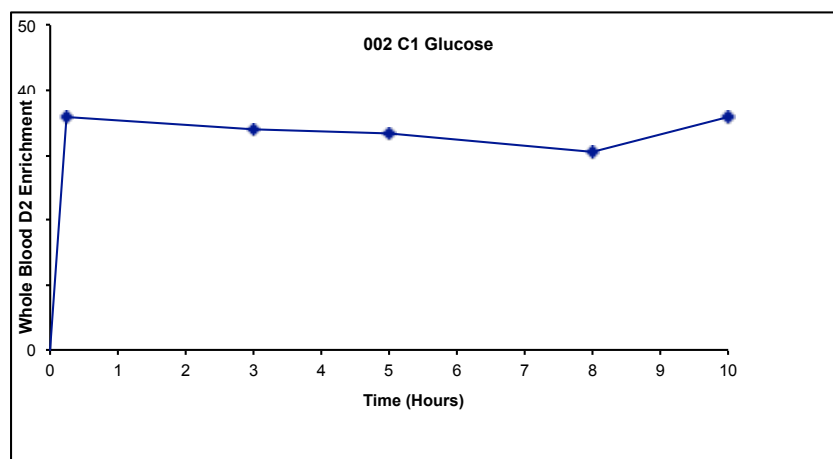


Figure 4.3 Example of deuterium enrichment of plasma glucose during labelling

Whole blood glucose deuterium enrichment following oral deuterated glucose labelling. The study participant received a priming dose of deuterated glucose at $t=0$, followed by aliquots at 30 minute intervals for 10 hours. Blood was sampled by finger-prick tests at pre-specified intervals post labelling. Deuterium enrichment was assessed by GC-MS.

b. Assessment of DNA deuterium enrichment.

- i. DNA was extracted using Qiagen Kit.
- ii. DNA digested to deoxyadenosine.
- iii. Digested DNA derivatised to PTFE derivative.
- iv. Deuterium enrichment analysed by GC-MS using selective monitoring of ions of M/Z 435, 437 (corresponding to M+0 and M+2).
- v. Ratio of $M+2/[M+0+M+2]$ of abundance matched samples used to calculate the enrichment of deuterated adenosine. Standard curves of known enrichment were used for calibration.
- vi. Analysis of ratios was repeated in triplicate.
- vii. The fraction of new DNA (f) was calculated by dividing DNA enrichment by the mean precursor enrichment

4.2.10 Ciclosporin Therapy

Ciclosporin (Neoral™) was commenced on Day 28 after labelling at a dose of 5mg/kg/day by mouth (PO), in a twice-daily (bd) dosing regime. Patients were reviewed twice weekly until CsA levels were stable (no dose adjustment for three consecutive doses) and weekly thereafter. Clinical reviews included physical examination and blood tests for full blood count, blood film, urea and electrolytes, liver function tests and CsA levels. EBV and CMV viral loads were measured on a weekly basis. CsA doses were adjusted to target levels of 150-200 ng/ml. CsA therapy was continued for a minimum of eight weeks; patients who responded to treatment had the option to continue therapy for a further four months. Stopping criteria included unacceptable toxicity, serious adverse events, a rise in creatinine > 100% from baseline, an increase in CMV/EBV levels to >100,000 copies per ml or the patient becoming pregnant. A final clinical review was performed two months after completion of treatment. Dr. Cuthill performed all clinical reviews, reviewed all results and advised patients on CsA dosing for patients recruited at King's College Hospital.

4.2.11 Translational Studies

Fifty milliliters of blood was drawn on day 14 post labelling and was processed at King's College London for further investigations into the mechanism of action of CsA. These studies included immunophenotyping of resting and stimulated CLL and CD4 T cells, assessment of resting and stimulated NFkB-p65 and NFAT-C2 translocation using image flow cytometry, analysis of T cell subsets and Gene Expression Profiling using the Illumina Bead Array platform. Methods were developed during the investigation of the *in-vitro* effect of CsA in CLL.

4.3 Results

4.3.1 Patient Characteristics

Five patients were enrolled across the two sites, four at King's College Hospital and one at University Hospital of Wales. All patients were men and the median age was 62 years. All patients had Binet Stage A CLL and only one patient had previously received treatment (Table 4.2). Patients all had normal cytogenetics except patient 004, who had a complex karyotype; this patient was also the only patient to have received prior therapy (Fludarabine, Cyclophosphamide and Rituximab). Patient 001 was withdrawn during Cycle 2 following the development of grade 2 atrial fibrillation. Patients 001-004 consented to the optional extra cycle of labelling to enable assessment of *intra*-patient variation in proliferation.

Table 4.2 Patient Characteristics

Trial Number	Age	Sex	Binet Stage	CD 38	IgV _H	FISH/cytogenetics	Previous Rx
001	69	M	A	7%	Mutated	No abnormality	Nil
002	53	M	A	66%	Mutated	No abnormality	Nil
003	65	M	A	>7%	Not assessed	Awaited	Nil
004	50	M	A	82%	Failed	Complex karyotype	FCR
005	62	M	A	9%	Mutated	Biallelic 13q del	Nil

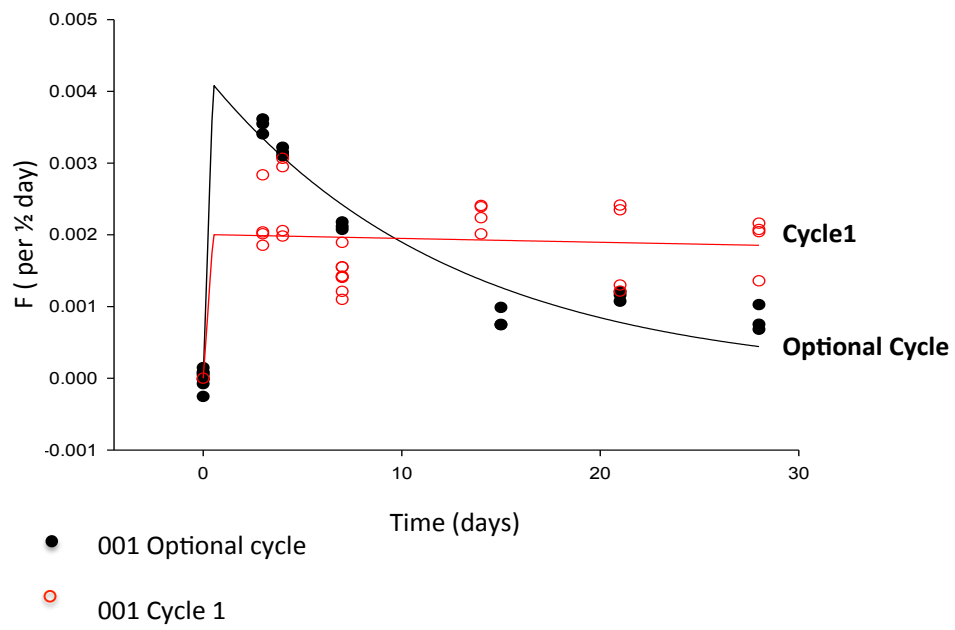
4.3.2 Primary End-point

The primary outcome measure, change in proliferation rate of CLL cells after four weeks was assessable in four patients. Modelled labelling curves, illustrating the fraction of deuterium labelled cells per day are shown in Figure 4.4 a-e. The turnover rates of CLL cells in the five subjects studied were generally very low and labelling curves tended to be flat indicating that the massive pool of leukaemic cells in the body has a very slow turnover rate. Nine studies were performed before CsA and the modelled mean proliferation rate of leukaemic cells was 0.353 ± 0.253 %/day (Table 4.3), equivalent to a doubling time of about 200 days. This is consistent with proliferation rates measured in deuterated water studies where overall proliferation rates were 0.1-1.76 %/day, and proliferation rates greater than 0.35 %/day were associated with progressive disease (Messmer et al., 2005). There was *intra*-subject variability in repeated pre-treatment data, mainly attributable to subjects 001 and 003. In the four studies performed after CsA treatment, proliferation rates were very similar to pre-treatment values, 0.254 ± 0.157 %/day, equivalent to a doubling time of about 270 days (Figure 4.4f).

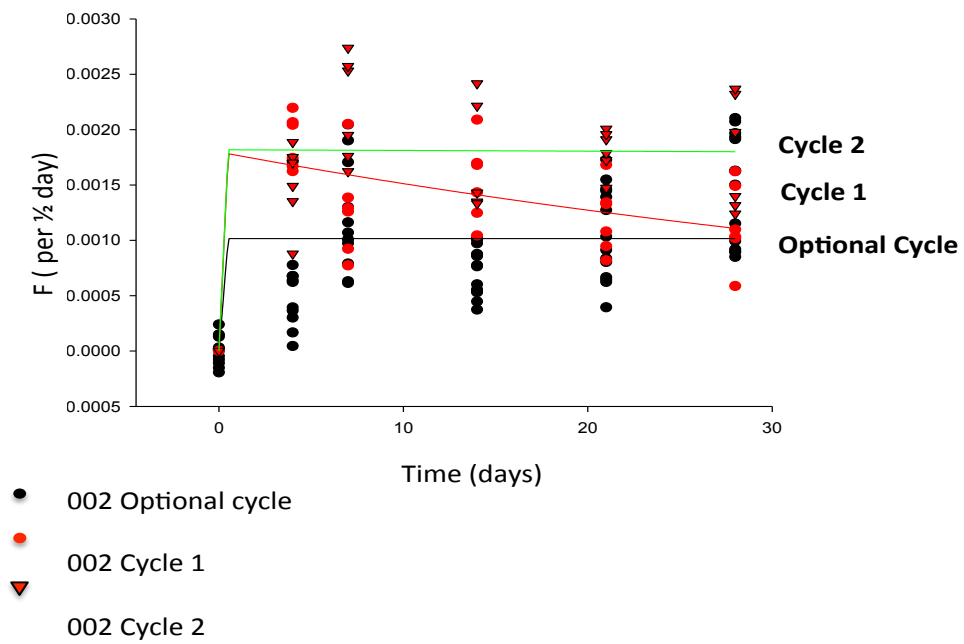
There was no difference in proliferation rate after four weeks' therapeutic CsA. Furthermore, there was evidence of background *intra*-patient variation in proliferation rates (Table 4.3, Figure 4.4).

In Patient 005, the proliferation rate appeared to increase in the presence of CsA. However, there was a slight reduction in the total lymphocyte count whilst CsA was being administered to this patient. One explanation for this observation may be that anergy was reversed in the presence of CsA i.e. BCR signalling was restored, allowing increased proliferation but a co-incidental increase in apoptosis. To interrogate this further, calcium-signalling capacity in response to BCR ligation pre- and during CsA therapy could be measured.

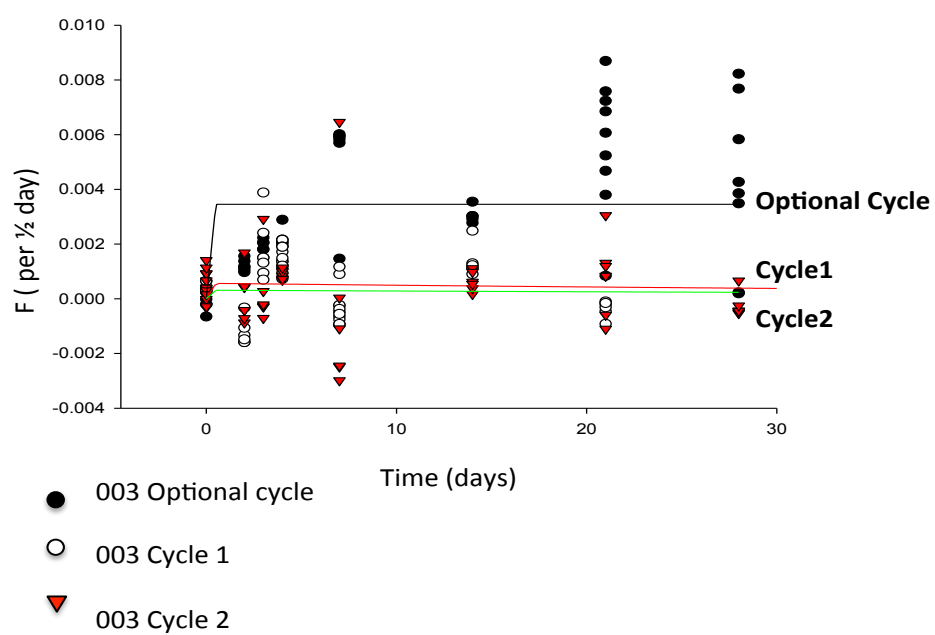
a. 001 – Optional Cycle + Cycle 1 Deuterium Enrichment



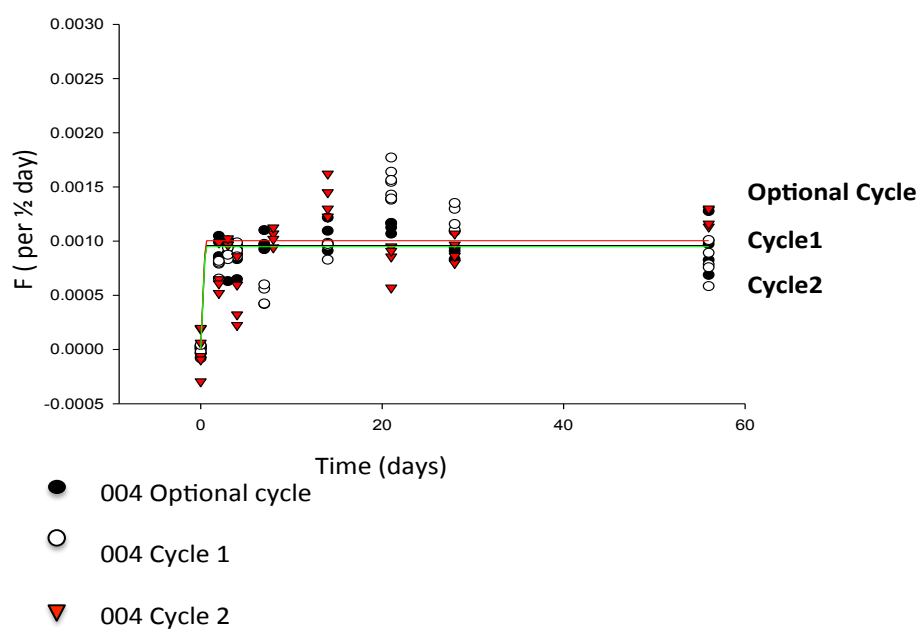
b. 002 – Optional Cycle, Cycle 1 and Cycle2 Deuterium Enrichment



c. 003 – Optional Cycle, Cycle 1 and Cycle 2 Deuterium Enrichment



d. 004 – Optional Cycle, Cycle 1 and Cycle 2 Deuterium Enrichment



e. 005 – Cycle 1 and Cycle 2 Deuterium Enrichment

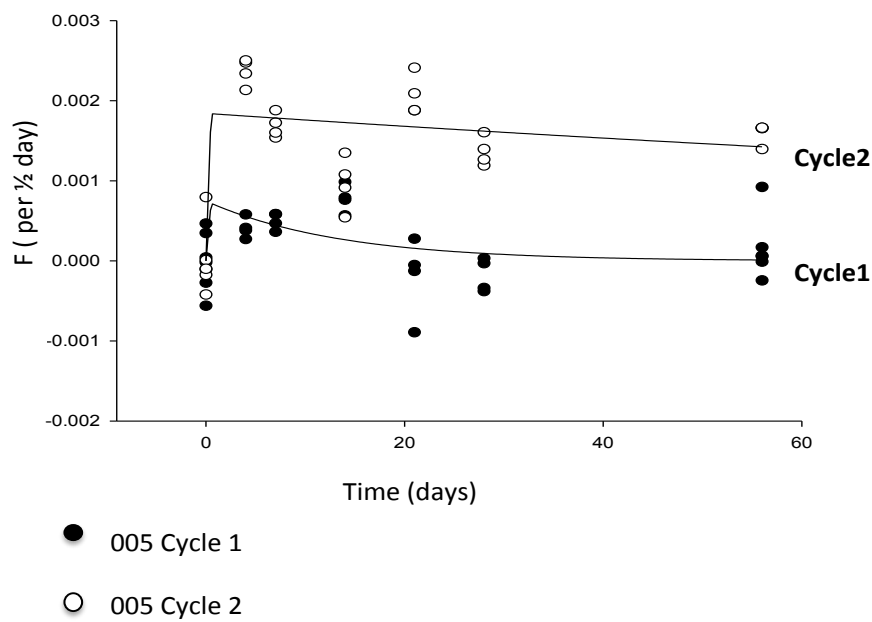


Figure 4.4 (a-e) Modelled deuterium labelling in CLL cells in patients recruited to CyCLLe

Patients 2-5 underwent 2 cycles of labelling. Cycle 1 was immediately pre-CsA and Cycle 2 was performed after 4 weeks therapeutic CsA. Patients 1-4 consented to an optional cycle of labelling to assess *intra*-patient variation in proliferation rates in the absence of CsA. Deuterium enrichment was measured by gas chromatography mass spectrometry and data was modelled by Prof. Macallan.

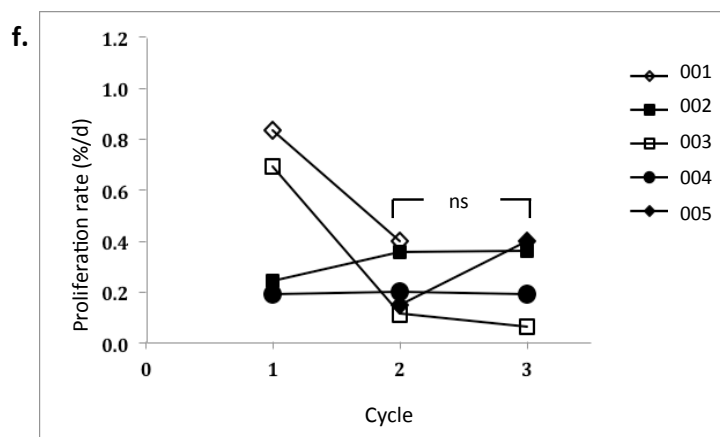


Figure 4.4 f Summary of proliferation rates for all patients

Each line represents a different patient. Patients 2-5 were labelled pre and post CsA. Patients 1-4 underwent an optional cycle of labelling to assess *intra*-patient variation in the absence of CsA.

Table 4.3 Proliferation Rate (% /day) as calculated by deuterium enrichment in purified CLL cells

Proliferation rate was measure by *in-vivo* labelling with deuterated glucose pre (C1) and post CsA (C2). An optional extra labelling cycle was performed in 4/5 patients.

Patient	Optional – P%/Day	C1- P%/Day	C2- P%/Day	Intra-patient variation P%/day (Optional vs C1)	Effect of CsA on P%/day (C1 v C2)
001	0.836	0.401		-0.435%	
002	0.243	0.358	0.364	+0.115%	+0.006%
003	0.691	0.113	0.063	-0.578%	-0.05%
004	0.192	0.201	0.190	+0.009%	-0.01%
005		0.147	0.399		+0.25%

4.3.3 Release and loss rates of CLL cells from circulation

Release and loss rates were calculated by measuring the fraction of labelled CLL cells at sequential time points post labelling (Table 4.4). As measurement of release rates required daily blood tests for the first week post labelling, it was an 'optional extra' procedure in the trial protocol. Measurement of time to maximum release of labelled cells into the circulation was measured in three patients at baseline and in one patient during CsA treatment. Time to maximum release rates was variable both between patients and within patients, with a range of 3-21 days pre-treatment. Time to maximum release was reduced from 21 to 14 days in the one patient assessed on treatment. Although no conclusions can be drawn from this isolated observation, shorter time to release in the presence of CsA is consistent with the *in-vitro* observation that CD69 expression is inhibited in the presence of the drug. CD69 expression has been shown to play a role in tissue retention of B-lymphocytes through down-modulation of S1P1 expression (Shiow et al., 2006).

Disappearance rates were difficult to estimate as enrichments were so low; in four measurements pre-CsA and one post-CsA no discernible loss of labelled cells occurred during the 28 day follow-up period.

Table 4.4 Release and Loss Rates of Labelled CLL cells

Mean time to maximum release of labelled CLL cells was assessed by drawing blood on a daily basis for the first week post labelling.

Cycle	Mean (95% CI) Time to Maximum release in days	Mean (95% CI) Loss rate of CLL cells
0	16.5 (95% CI 2.17, 30.82)	2.02 (95% CI -4.42, 8.46)
1	9.2 (95% CI -3.98, 22.38)	2.16 (95% CI -1.62, 5.93)
2	7.25 (95% CI -0.256, 14.76)	0.36 (95% CI -0.38, 1.10)

4.3.4 Toxicity of ciclosporin

Patients were subject to twice weekly assessments until the CsA dose had remained unchanged for three consecutive doses. Trough levels were targeted between 150-200ng/ml. The median time in range was 46.5 % (30-50 %). A median of 83.5 % (66-86 %) of all readings were within a 25 % margin of the therapeutic range (Figure 4.5). Toxicity assessments included physical examination, biochemical assessment of renal function, liver function, electrolytes and glucose. Patients were screened for EBV and CMV reactivation with weekly viral load monitoring.

Adverse Events listed in (Table 4.5) were mainly grade 1-2 and included headache, myalgia, nausea, paraesthesia, tremor. In one patient, headache was considered to be severe (grade 3). There were three episodes of infection (grade 1-2). One patient (001) was withdrawn from the study when he developed atrial fibrillation whilst taking CsA. Two days later, he was admitted to hospital with a myocardial infarction (grade 3) and underwent coronary artery bypass grafting. The atrial fibrillation and myocardial infarction were considered to be serious adverse events unrelated to the study medication; there were no electrolyte abnormalities at the onset of atrial fibrillation.

Laboratory abnormalities were mostly grade 1 and included rise in alkaline phosphatase in 3 /5 patients, rise in bilirubin in 2/5 patients, rise in creatinine in 5 (grade 1 x 4, grade 2 x 1) and grade 1 hypomagnasemia in 4 patients. These were predictable toxicities of CsA.

One patient was found to have reactivated EBV (grade 1) but remained asymptomatic and the viraemia resolved spontaneously.

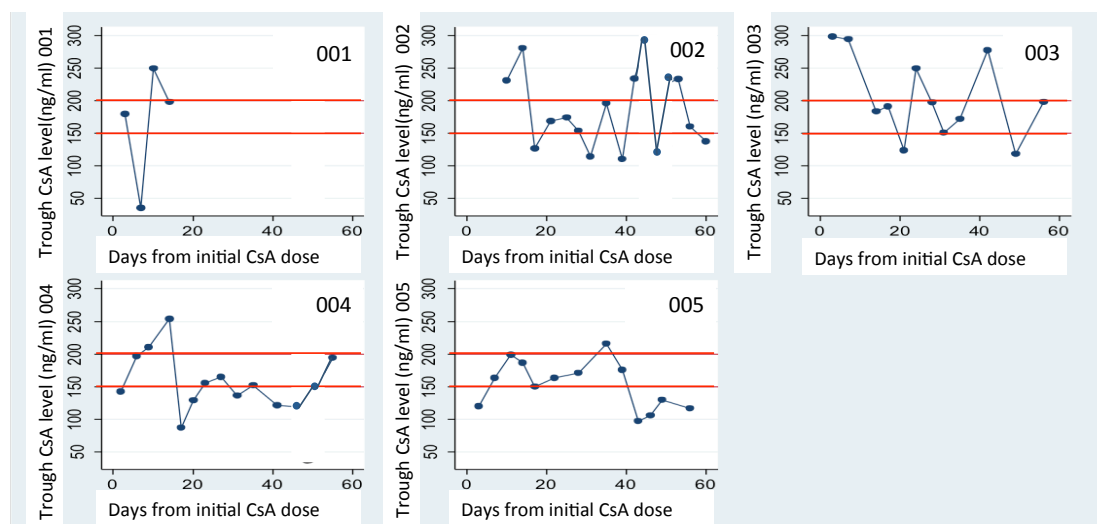


Figure 4.5 CsA trough levels in CyCLLe subjects

CsA levels were measured in the Clinical Pathology Accredited (CPA) accredited laboratories at KCH and UHW. CsA levels were titrated to a therapeutic range of 150-200 ng/ml by Dr. Cuthill.

Table 4.5 Adverse Events

Adverse Event	Grade 1 N (%)	Grade 2 N (%)	Grade 3 N (%)
Nausea	5 (100 %)	0	0
Infection	0	2 (40 %)	0
Myalgia	4 (80 %)	0	0
Headache	4 (80 %)	0	1 (20 %)
Tremor	2 (40 %)	0	0
Paraesthesia	3 (60 %)	0	0
Cardiac	0	1 (20 %)	1
Laboratory:	3 (60 %)	0	0
Alkaline phosphatase			
Laboratory:	1 (20 %)	0	0
Bilirubin			
Laboratory:	4 (80 %)	1 (20 %)	0
Creatinine			
Laboratory:	4 (80 %)	0	0
Magnesium			
Laboratory:	1 (20 %)	0	0
EBV VL			

4.3.5 Clinical response to ciclosporin

Response rate to CsA was measured on D56 in four patients using the Hallek Criteria for Response Assessment (Hallek et al., 2008). All patients had stable disease after eight weeks of CsA therapy. On review of serial lymphocyte counts (Figure 4.6), 4/5 patients were observed to develop an increase in peripheral blood lymphocyte count immediately after initiation of CsA therapy that resolved over subsequent weeks. This finding suggests that there is enhanced release of malignant cells from the tumour microenvironment into the peripheral circulation in the presence of CsA.

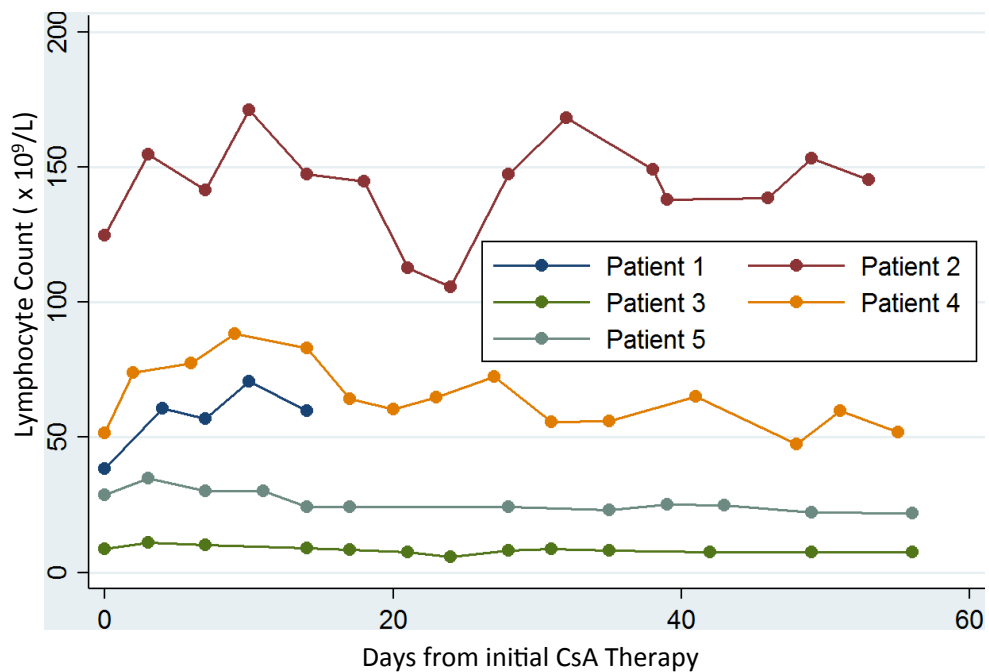


Figure 4.6 Serial lymphocyte counts during CsA therapy

Peripheral blood lymphocyte counts were monitored in all patients from the time of baseline assessment and throughout CsA therapy.

4.3.6 Translational Studies - Peripheral blood derived CD4 T cells show trend towards reduced activation following four weeks of ciclosporin therapy

The CyCLLe study was based on the hypothesis that inhibition of activation of CD4 T cells in CLL may reduce CLL activation and proliferation *in-vivo*. It was therefore critical to investigate whether the T cell suppressive effect of CsA seen *in-vitro* is relevant *in-vivo*. The activation status of CD4 T cells was measured in fresh CD4 T cells negatively selected from PBMCs pre treatment with CsA and after 4 weeks of CsA therapy (blood drawn 12 hours after last dose). The activation of CD4 T cells was assessed at rest and following activation with anti-CD3 and anti-CD28 to investigate if CsA therapy affects the potential of peripheral blood CD4 T cells to be activated in the appropriate environment. Activation status was assessed by flow cytometric measurement of CD25, CD69, and CD40L expression. *In-vitro* investigation into the effect of CsA on CD4 T cell activation by anti-CD3 and anti-CD28 had shown that CD69 and CD40L are inhibited in the presence of CsA and that there is a trend towards reduced CD25 expression. Although expression of activation-markers in the resting-state was unchanged during CsA therapy, there was a trend towards reduced capacity to be activated by CD3/28 ligation. In all three patients, expression of CD25 and CD69 by activated CD4 T cells was reduced on treatment, and this pattern held for CD40L expression in two of three patients. As *in-vivo* assessment of T cell activation was only possible in three patients, the results did not reach statistical significance (Figure 4.7).

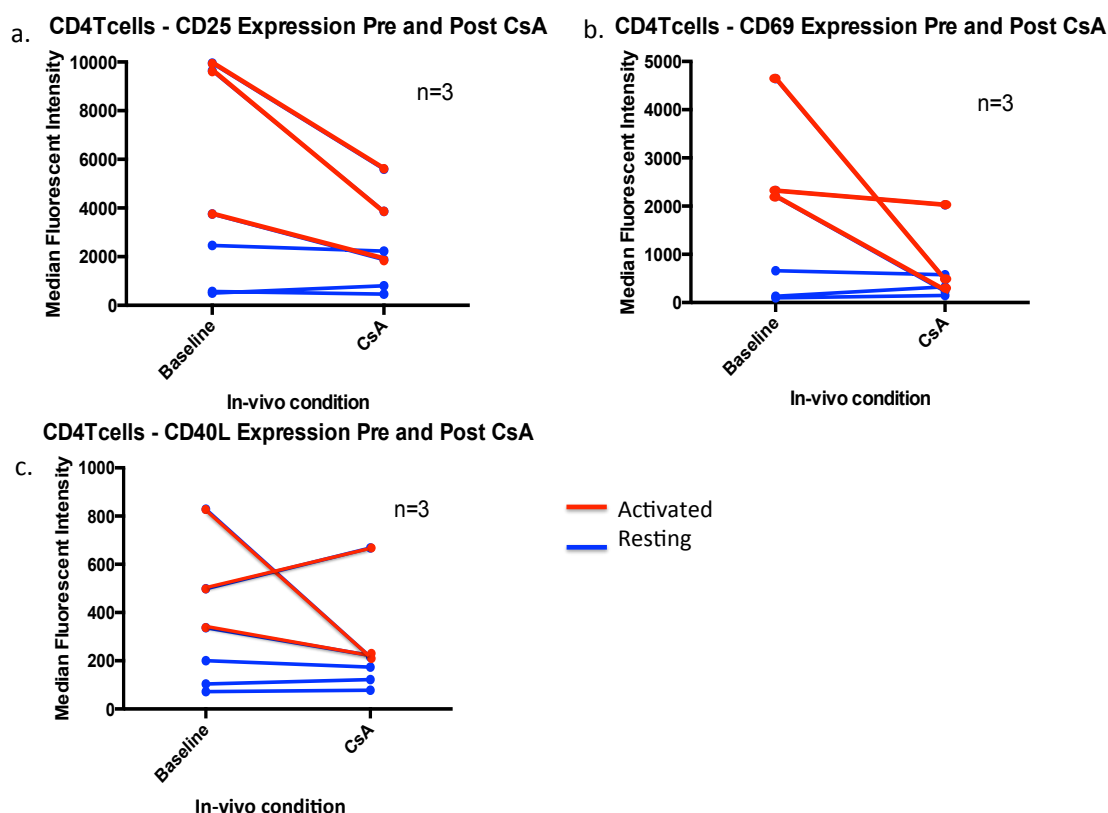


Figure 4.7 Resting and activated CD4 T Cell expression of CD25, CD69 and CD40L pre and post CsA therapy

Expression of CD25, CD69 and CD40L was flow cytometrically measured on fresh, viable CD4 T cells before and after 4 weeks therapeutic CsA. Expression was measured in both the resting and activated state. Activation was achieved using anti-CD3 coated plates and un-bound anti-CD28.

4.3.7 Translational Studies – Peripheral blood derived CLL cells show mixed response to four weeks of *in-vivo* ciclosporin therapy

Having shown that CD4 T cell activation is suppressed following *in-vivo* CsA therapy, and on the basis that *in-vitro*, this lead to reduced CLL cell activation, we went on to measure CLL cell activation status in both the resting and CD40L-stimulated state pre and post CsA treatment. Expression of CD25, CD38 and CD69 was measured in freshly collected, viable CLL cells. CD38 is a Type II transmembrane glycoprotein that participates in signal transduction necessary for activation and proliferation of B lymphocytes (Kumagai et al., 1995)

To test the effect of *in-vivo* CsA on the ability of CLL cells to respond to CD40L activation, negatively selected CLL cells were stimulated with CD40L transfected fibroblast-coated plates. Expression of CD25 and CD38 showed a mixed response; however, expression of CD69 by stimulated CLL cells was consistently suppressed following CsA treatment. Again, this result did not reach statistical significance due to the small number of patients investigated (Figure 4.8). This observation is in contrast to the finding that CD69 expression by CLL cells activated with CD40L *in-vitro* was not inhibited by CsA. The difference between *in-vitro* and *in-vivo* findings may relate to the fact that CsA was administered for four weeks pre-activation with CD40L transfected fibroblasts in the *in-vivo* study whilst CLL cells were preconditioned with CsA for 60 minutes in the *in-vitro* assay, allowing time for the gene expression of CLL cells to be altered.

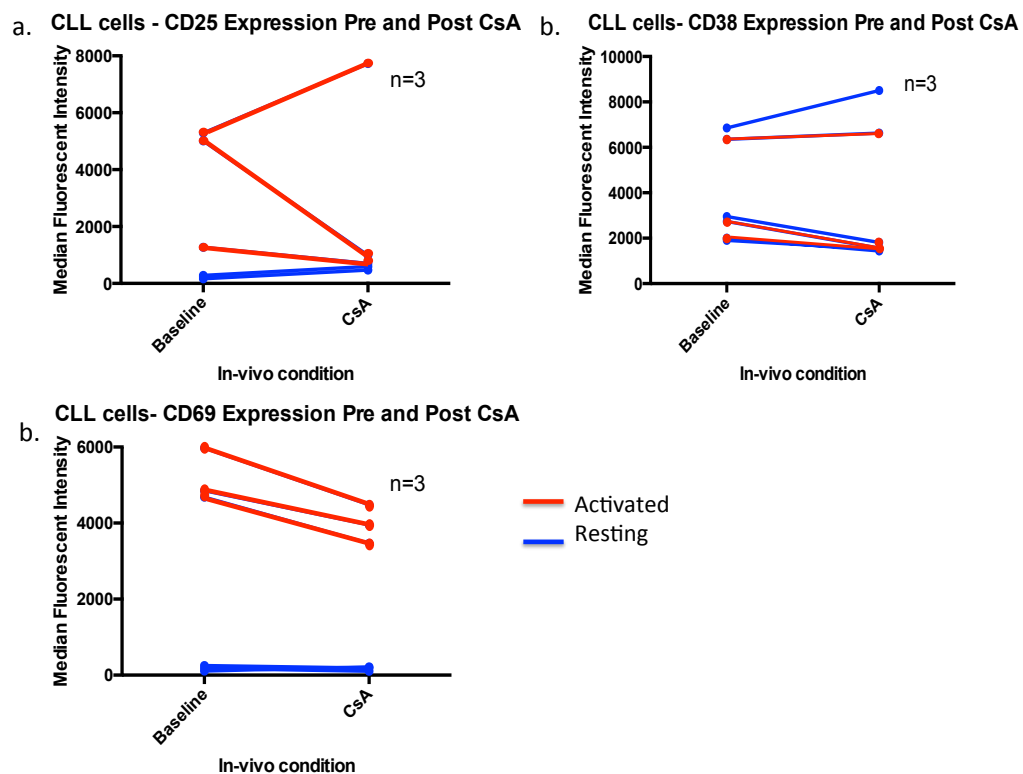


Figure 4.8 Resting and activated CLL cell expression of CD25, CD38 and CD69 pre and post CsA Therapy

Expression of CD25, CD38 and CD69 was flow cytometrically measured on fresh, viable CLL cells before and after 4 weeks therapeutic CsA. Expression was measured in both the resting and activated state. Activation was achieved using CD40L transfected fibroblasts. The sample size eliminated the potential for statistical analysis.

4.3.8 *In-vivo* ciclosporin suppresses CLL cell activation by autologous CD4 T cells

Having demonstrated a trend towards suppression of CD4 T cell activation following *in-vivo* CsA therapy, the effect of *in-vivo* CsA on the ability of CD4 T cells to activate CLL cells was next investigated. PBMCs were harvested pre and post treatment with CsA. CD4 T cells were isolated and activated with anti-CD3 and anti-CD28 for 12 hours before re-combining with purified CLL cells for 24 hours. No additional CsA was added to the culture medium. In the post *in-vivo* CsA co-culture, CD25 expression at 24 hours was reduced by a mean of 37 %, CD38 expression was reduced by a mean of 36 % and CD69 expression was reduced by a mean of 16.4 %. The sample size eliminated the potential for statistical analysis. These findings suggest that following *in-vivo* CsA, CD4 T cells may be less able to activate CLL cells.

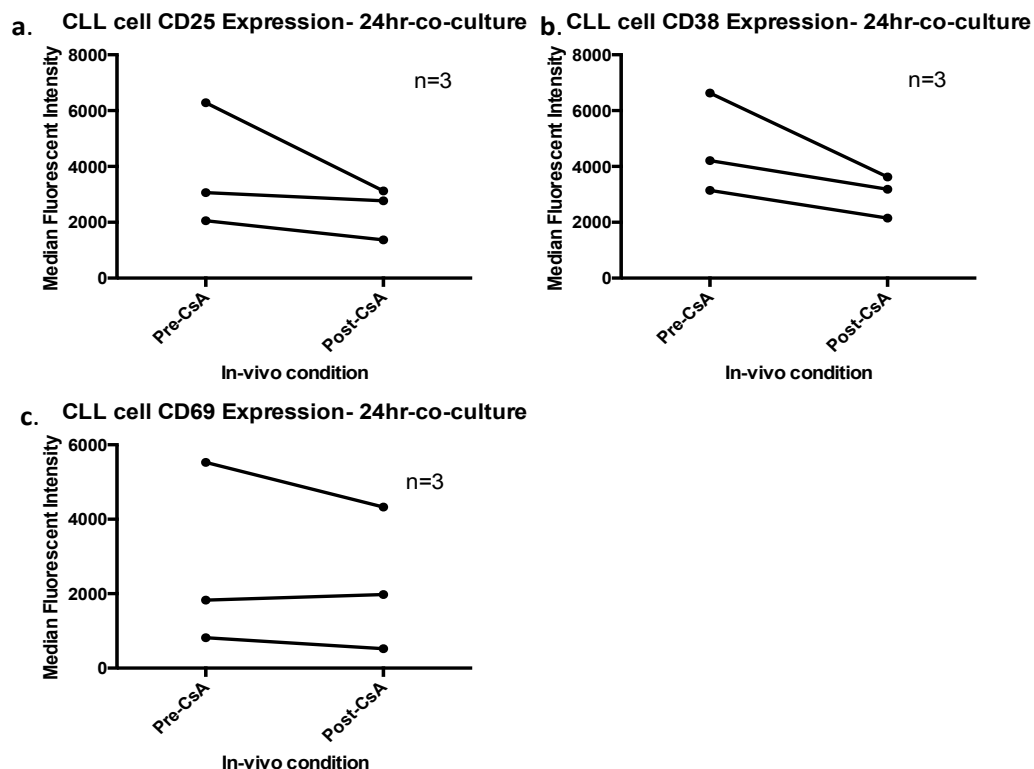


Figure 4.9 CLL cell activation pre and post *in-vivo* CsA following co-culture with activated autologous CD4 T cells

CLL cells from patients pre and post CsA treatment were co-cultured for 24 hours with CD4 T cells that had been activated with anti-CD3 / anti-CD28. Activation was assessed by flow-cytometric measurement of CD25, CD38 and CD69 expression.

4.3.9 Effect of *in-vivo* ciclosporin on baseline transcription factor activation in CLL and CD4 T cells

In-vitro studies that utilised image flow cytometry to assess the effect of CsA on CD4 T cell and CLL cell transcription factor activation demonstrated that CsA inhibited nuclear translocation of NFAT-C2 and NFκB-p65 in CD4 T cells activated with PMA and Ionomycin. Furthermore, NFAT-C2 and NFκB-p65 nuclear translocation in CD40L activated CLL cells could not be overcome by CsA. To investigate the effect of *in-vivo* CsA therapy on transcription factor activation, NFAT-C2 and NFκB-p65 localisation was assessed in freshly collected CD4 T cells and CLL cells. There was no significant difference between pre- and post-treatment transcription factor localisation in resting CLL and CD4 T cells.

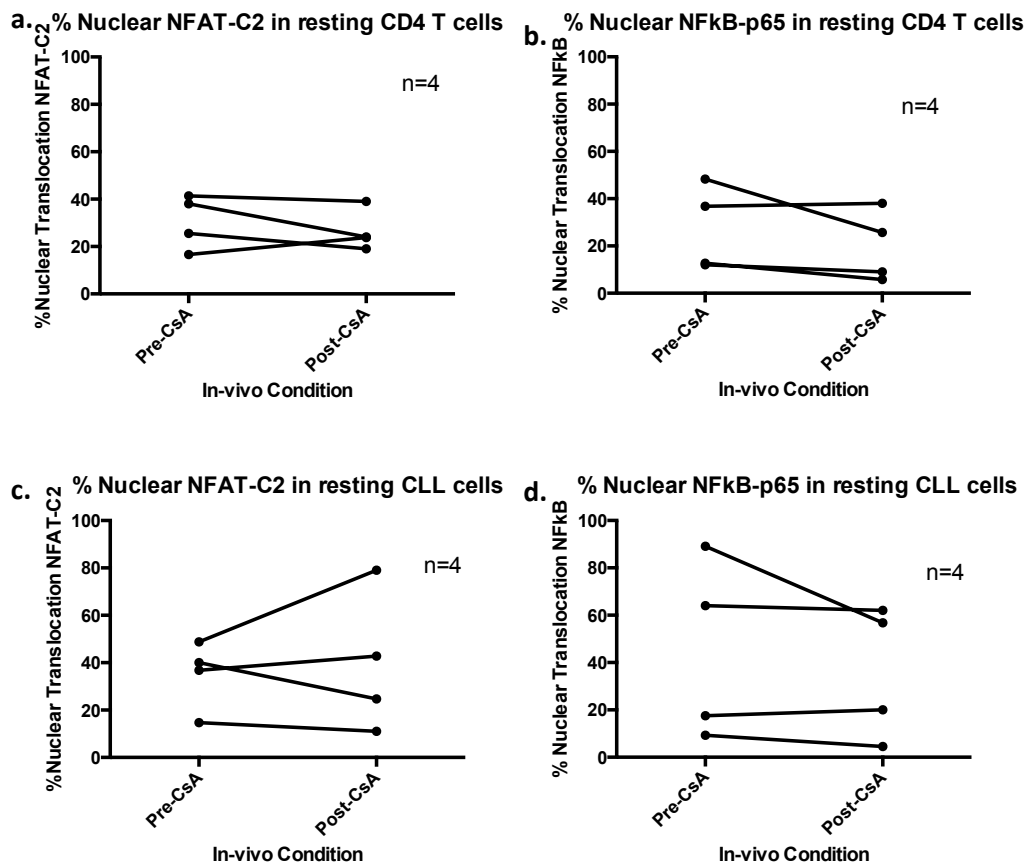


Figure 4.10 Nuclear NFAT-C2 and NFκB-p65 in resting CLL and CD4 T cells pre and after 4 weeks CsA therapy

CLL/ CD4+ T cells were gated on and the degree of nuclear translocation of NFκB-p65 and NFAT-C2 was calculated using a similarity score between the nucleus (DAPI) and the transcription factor (FITC).

4.3.10 There is a trend towards reduced transcription factor activation in CLL and CD4 T cells following *in-vivo* ciclosporin therapy

In view of the observation that CsA inhibits nuclear translocation of NFAT-C2 and NFκB-p65 in activated CLL CD4 T cells *in-vitro*, the effect of *in-vivo* CsA on transcription factor activation was measured. Following 4 weeks of *in-vivo* CsA therapy, CD4 T cells were isolated and activated for 30 minutes with PMA and Ionomycin. Figure 4.11a-b shows that there is a trend towards reduced nuclear translocation of NFAT-C2 and NFκB-p65, consistent with the *in-vitro* findings.

We then went on to investigate the effect of *in-vivo* CsA on NFAT-C2 and NFκB-p65 in CLL cells activated with CD40L transfected fibroblasts. Although the *in-vitro* studies demonstrated no-effect of CsA on CD40L mediated transcription factor activation in CLL cells, and although there was no discernible effect on resting CLL cells, there was a reduction in nuclear NFAT-C2 and NFκB-p65 in 2/3 patients assessed (Figure 4.11c-d). Assessment of transcription factor activation was only measureable in 3 patients on the CyCLLe study, therefore findings did not reach statistical significance.

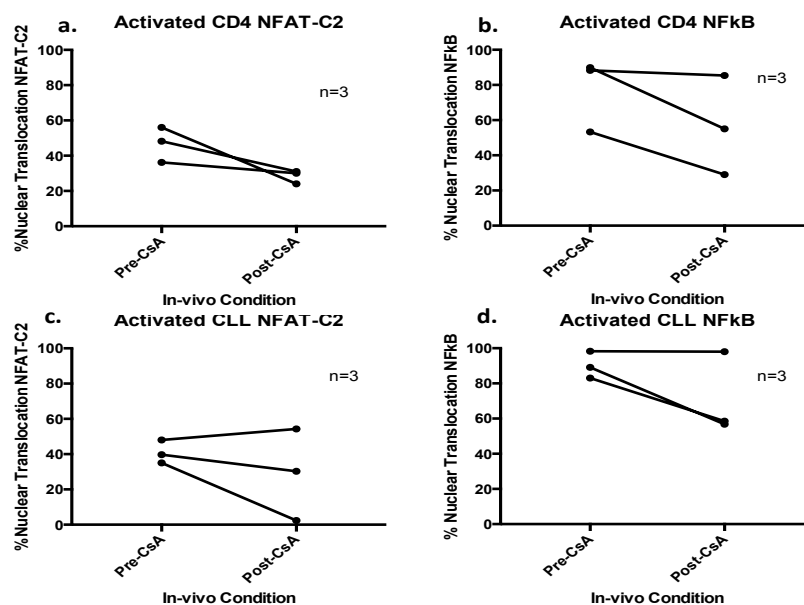
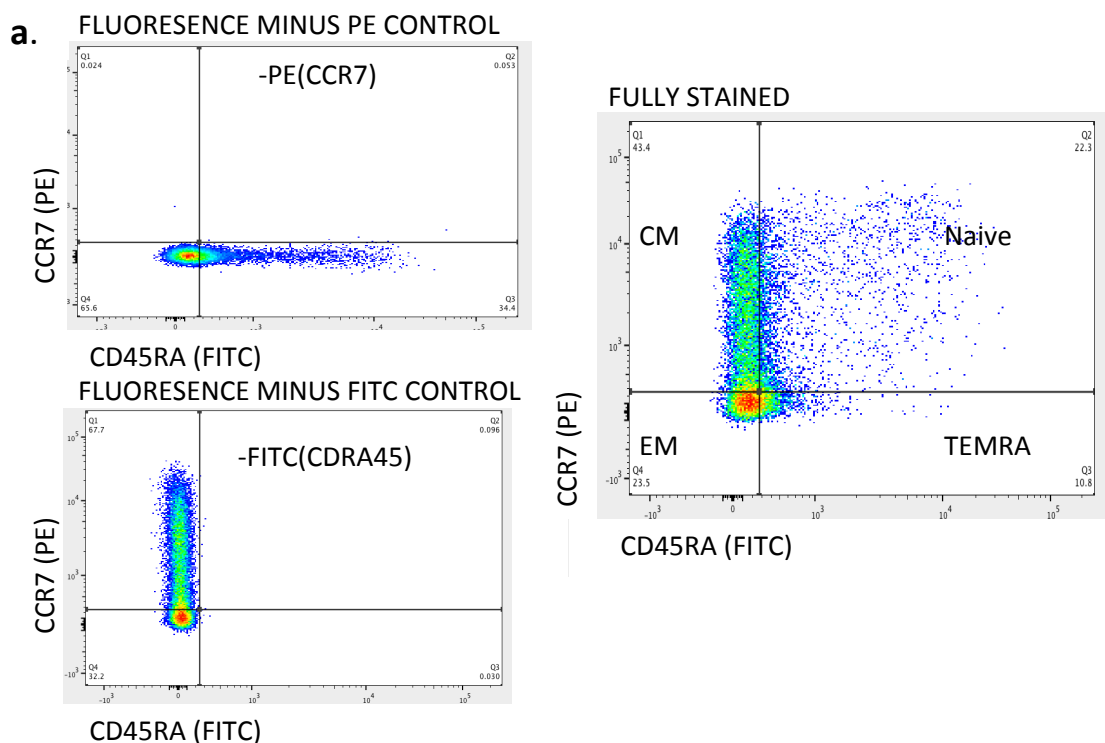


Figure 4.11 Nuclear NFAT-C2 and NFκB-p65 in activated CLL and CD4 T cells pre and after 4 weeks CsA therapy

CD4 T cells were activated using PMA and Ionomycin and CLL cells were activated using CD40 L transfected fibroblasts. CLL/ CD4+ T cells were gated on and the degree of nuclear translocation of NFκB-p65 and NFAT-C2 was calculated using a similarity score between the nucleus (DAPI) and the transcription factor (FITC).

4.3.11 Ciclosporin has no effect on T cell subsets following *in-vivo* therapy

A number of abnormalities of T cell number and function have been reported in CLL; it has been shown that there is skewing of the normal CD4: CD8 ratio with expansion of CD8 T cells and accumulation of terminally differentiated effector memory T cells with a relative paucity of naïve precursors (Nunes et al., 2012). We therefore investigated whether 4 weeks CsA therapy had an impact on these subsets in patients with CLL. PBMCs were collected pre- and after 4 weeks CsA therapy. Subsets were assessed by flow cytometry, gating on single, viable T cells according to the expression of CCR7 and CD45RA, classifying T cells as Naïve, CM (central memory), EM (effector memory) EMRA (terminally differentiated effector). Gates were set using 'fluorescence minus one' controls, as illustrated in Figure 4.12 a. Figure 4.12 b illustrates pie-charts of CD4 and CD8 subsets pre and post CsA for each patient that are summarized in Figure 4.11 c-d. CD8 T cells were particularly skewed towards a terminally differentiated effector phenotype (TEMRA). T cell subsets remained stable after 4 weeks of *in-vivo* CsA therapy.



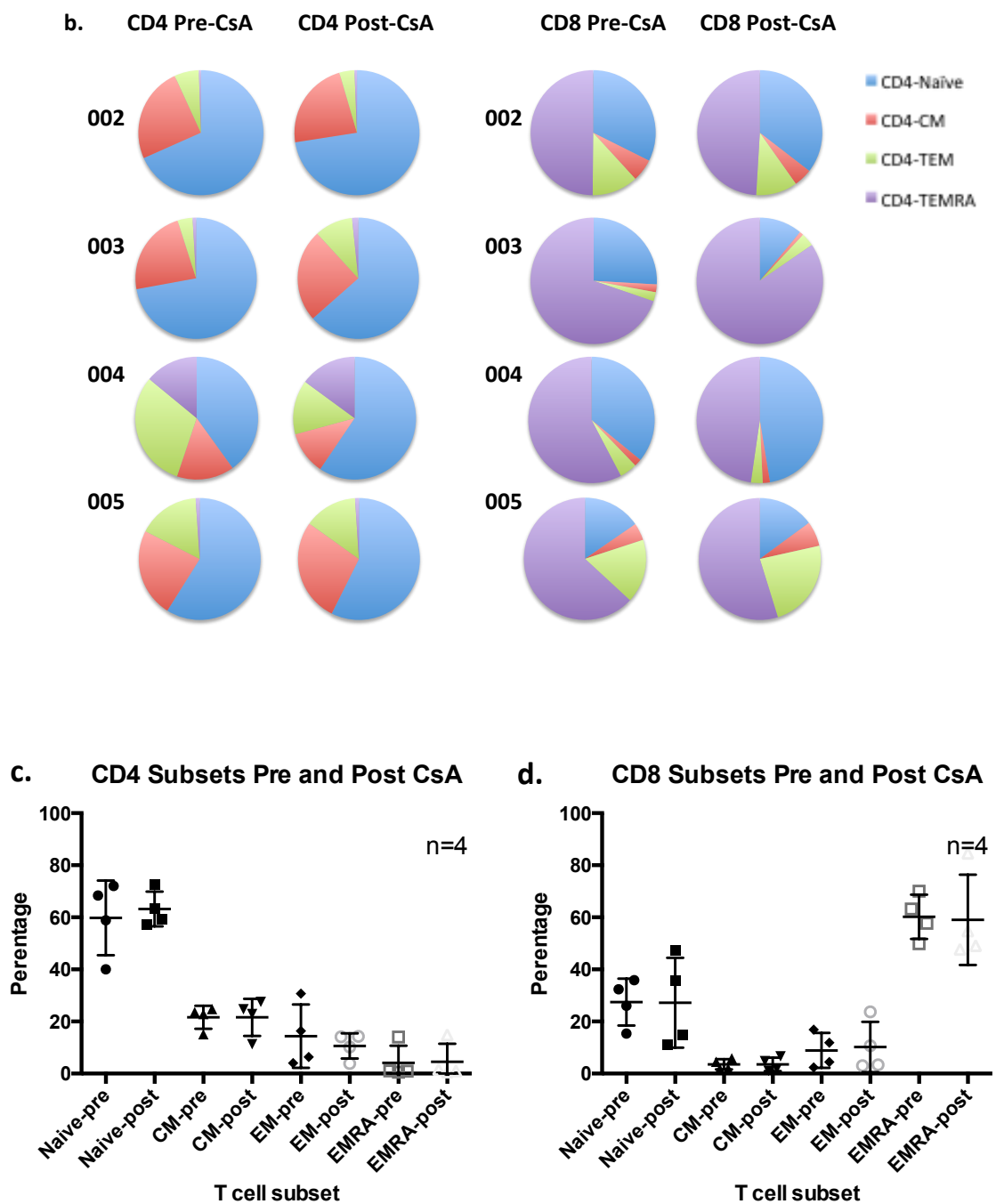


Figure 4.12 (a-d) T cell subsets assessed pre and post 4 weeks of *in-vivo* CsA therapy

T cell subsets were assessed in 4 patients pre and after 4 weeks CsA therapy.

a. Sub-sets were measured by flow cytometry, gating on single, viable CD4/ CD8 T cells according to the expression of CCR7 and CD45 RA and classifying T cells as Naïve, CM (central memory), EM (effector memory) EMRA (terminally differentiated effector). Gates were set using FMO controls.

b. Pie charts illustrating CD4 and CD8 T cell sub-sets in each participant pre and post 4 weeks *in-vivo* CsA therapy.

c. Summary of CD4 and CD8 T cell sub-sets pre and post 4 weeks *in-vivo* CsA therapy.

4.3.12 Translational Studies – PD-1 Expression by CLL CD4 and CD8 T cells is not sensitive to 4 weeks *in-vivo* ciclosporin therapy

CLL CD4 and CD8 T cells have been shown to have increased expression of PD-1, a marker of T cell exhaustion, compared to age-matched controls (Brusa et al., 2013) and it has been proposed that PD-1 ligation by CLL cells plays a role in immune-evasion (Brusa et al., 2013; Riches et al., 2013). Furthermore, PD-1 expression has been shown to be under transcriptional control of NFAT-C1 and sensitive to CsA (Oestreich, Yoon, Ahmed, & Boss, 2008). We therefore measure PD-1 expression on peripheral blood T cells before and after 4 weeks CsA therapy (Figure 4.13). PD-1 expression was not significantly affected by the presence of CsA (n=4).

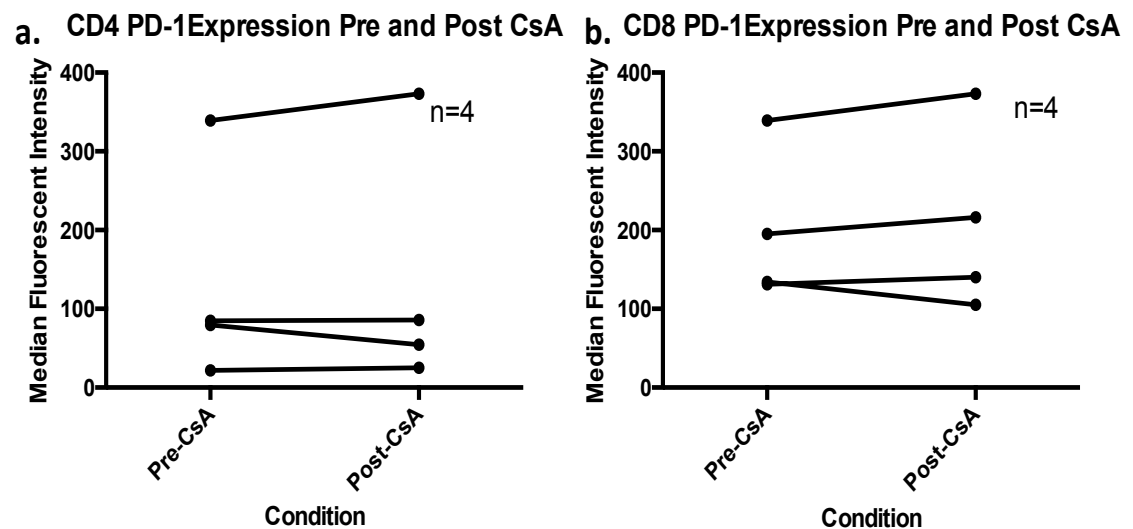


Figure 4.13 PD-1 Expression before and after 4 weeks of *in-vivo* CsA therapy

PD-1 expression by viable, peripheral blood CD4 and CD8 T cells was measured by flow cytometry pre and after 4 weeks therapeutic CsA in 5 patients with CLL.

4.3.13 Translational Studies – Effect of *in-vivo* ciclosporin on gene expression profile of CLL and CD4 T cells

Gene expression profiling using microarray technology was employed to compare gene expression in purified CD4 T cells and CLL cells pre and after four weeks of CsA therapy. It was crucial to assess gene expression *in-vivo* in view of the complex interactions between CLL and CD4 T cells that cannot be accurately replicated *in-vitro*.

Briefly, cells were purified by fluorescence activated cell sorting (FACS) ensuring purity > 95 %. RNA was extracted using Qiagen RNeasy mini-kit. The quantity and quality of RNA was assessed using the NanoDrop 2000 (Thermo Scientific) spectrophotometer. A minimum of 150ng/mcl was required for hybridization; this was achieved with purified CLL cells pre and post treatment from all 5 patients but from CD4 T cells in only 3 patients. Aliquots of RNA were transferred to the Genomics core at the BRC, where gene expression was assessed using Illumina® Microarray Bead Chip technology (Chapter 2.7). RNA hybridization to the BeadChip microarray was assessed using Illumina® iScan technology. Comparison of gene expression was performed using Genome Studio Software. Bonferroni correction and false discovery rate adjustment were applied to correct for multiple hypothesis testing.

Following Bonferroni correction, there was reduced transcription of two genes in CD4 T cells post CsA therapy; *DDIT4* (DNA damage inducible transcript 4) ($p= 3.30^{-7}$) and *PTGER4* (Prostaglandin E receptor 4) ($p = 9.57^{-7}$). Using the less stringent test of False Discovery Rate, *SNORA12* (small nucleolar RNA) was also suppressed in CD4 T cells in the presence of CsA.

DDIT4 encodes DDIT4, which acts as a negative regulator of mTOR, a serine/threonine kinase that regulates a variety of cellular functions such as growth, proliferation and autophagy. It has not previously been implicated in the mechanism of action of CsA.

PTGER4 encodes prostaglandin E receptor 4 (EP4), a g-protein coupled receptor that has a pro-inflammatory mechanism of action in T cells, implicated in cancer and auto-immunity (Sreeramkumar, Fresno, & Cuesta, 2011). EP4 leads to Th1 and Th17 skewing that can be blocked by EP4-specific antagonism, suppressing Th1 and Th17 expansion in the lymph-node of an animal model of inflammation (Yao et al., 2009). Little is

understood about the regulation of *PTGER4* transcription in T cells and again, its inhibition has not previously been implicated in the mechanism of action of CsA. *SNORA12* encodes a small nucleolar RNA that is involved in RNA processing and its role has not previously been reported to be a target of CsA therapy.

The results of the gene expression studies should be tested by performing reverse transcription quantitative polymerase chain reaction (RT-qPCR) of *DDIT4*, *PTGER4* and *SNORA12* genes. However, there was an insufficient quantity of CD4 T cells to extract additional RNA so it was not possible to further validate microarray results.

4.4 Discussion

The purpose of the CyCLLe study was to investigate whether manipulation of the tumour microenvironment, using the calcineurin inhibitor CsA to suppress CD4 T cell activation, inhibits the proliferation rate of CLL cells *in-vivo*. *In-vitro* studies demonstrated that in CLL, CsA inhibits the activation of CD4 T cells and that when co-cultured with CLL cells, CsA-treated autologous CD4 T cells were less able to activate CLL cells. There was an additional direct effect of CsA on CLL cells, notably inhibiting CD69 expression which plays a role in retention of CLL cells in the lymph-node compartment (Shiow et al., 2006; Sic et al., 2014).

The study aimed to investigate the effect of CsA in patients with early stage adverse-risk CLL in whom, despite the more progressive nature of the disease, there is currently no known intervention that improves clinical outcome.

The initial study was designed to intensively investigate the *in-vivo* effect of CsA in a small cohort of patients, with a plan to expand to a larger cohort study with clinical end-points if there was a measurable reduction in the proliferation rate of CLL cells. Unfortunately, despite the convincing effect of CsA *in-vitro*, the trial failed to recruit fully, making it difficult to draw conclusions and a larger cohort study could not be justified.

The proliferation rate of CLL cells was measured using *in-vivo* labelling with deuterated glucose, the first time that this technique has been employed to assess the effect of drug treatment. Pre-treatment proliferation rates were low (mean = 0.35 %/day), similar to proliferation rates in patients with no indication for treatment, reported using deuterated water (Messmer et al., 2005). Spontaneous *intra*-patient variation in proliferation was observed in 4 patients who consented to the optional extra labelling cycle; this was considered to be attributable to low enrichment rates resulting from a large pool of non-proliferating CLL cells. There was no measurable reduction in proliferation of CLL cells after 4 weeks of CsA therapy and there was no clinical response observed after 8 weeks of treatment; all patients had stable disease.

Four of five patients were observed to develop a lymphocytosis upon commencement of CsA therapy, suggestive of an effect of CsA on CLL-cell interaction with the tumour

microenvironment. This may be explained by the observation that *in-vitro*, CsA inhibits CD69 expression by CLL cells activated by both autologous CD4 T cells and through the BCR. CD69 expression by CLL cells plays a role in tissue-retention by down-regulating S1P1 expression that is required for egress of tumour cells from the lymph-node compartment (Sic et al., 2014). Furthermore, the expression of CD69 by CD40L-activated CLL cells appeared to be inhibited following *in-vivo* CsA, however it was not possible to analyse for statistical significance due to the small number of patients assessed. An acute lymphocytosis related to the redistribution of CLL cells from the lymph-node compartment into the peripheral blood is a recognised feature in patients treated with drugs that inhibit BCR signalling (Herman, Niemann, et al., 2014b). Notably, BTK inhibition with ibrutinib leads to a rapid reduction in CD69 expression and a reduction in proliferation of peripheral blood CLL cells (measured by Ki67 expression) *in-vivo* (Herman, Mustafa, et al., 2014a). However, down-regulation of CXCR4 signalling is thought to contribute to the egress of CLL cells in patients treated with ibrutinib (Herman, Niemann, et al., 2014b). Expression of this chemokine receptor should be measured in the context of CsA therapy, particularly as it is under the transcriptional control of NFAT3 and has been shown to be sensitive to CsA in an *in-vitro* model (Y. H. Huang, Sojka, & Fowell, 2012).

Patients tolerated CsA treatment well, with the commonest side effects being grade 1 headache, nausea and myalgia. Importantly, there were no severe infections. Patient 001 was withdrawn from the study after one week of CsA therapy, following the development of atrial fibrillation. Two days after discontinuing treatment, he went on to sustain a myocardial infarction. These events were deemed to have been independent of the treatment.

Translational studies revealed no significant difference in the activation status of unstimulated CD4 and CLL cells following *in-vivo* CsA therapy. However, post-treatment, there was a trend towards reduced responsiveness of CD4 T cells to stimulation with anti-CD3/ anti-CD28. In order to replicate CD4 T cell: CLL interactions in the tumour microenvironment, CLL cells were co-cultured with activated autologous CD4 T cells before and during CsA treatment. In the co-cultures performed during *in-vivo* CsA treatment, there was a trend towards inhibition of CLL cell activation, suggesting that inhibition of CD4 T cell activation translates into reduced CLL cell

activation. Additionally, there was a trend towards reduced CD69 expression by CLL cells activated with CD40L during *in-vivo* therapy, suggesting that CsA may reduce the response of CLL cells to T cell activation.

In view of the previously described mechanism of action of CsA, activation of transcription factors NFκB-p65 and NFAT-C2 was assessed using image flow cytometry to measure nuclear translocation in CD4 T cells and CLL cells pre and during *in-vivo* CsA. Although there was no overall difference in transcription factor activation in unstimulated CD4 T cells, there was a trend towards reduced NFκB-p65 and NFAT-C2 nuclear translocation following PMA and Ionomycin activation. Furthermore, although CsA did not inhibit NFκB-p65 and NFAT-C2 nuclear translocation in CD40L activated CLL cells *in-vitro*, there was a reduction in transcription factor activation in response to CD40L stimulation in two of three patients assessed during *in-vivo* CsA therapy. An explanation for this finding is that CLL cells may have altered gene expression and therefore altered sensitivity to CD40L activation following four weeks of CsA treatment.

One patient (005) exhibited reduced nuclear translocation of NFκB-p65 in CD4 T cells and reduced NFκB-p65 and NFAT-C2 nuclear translocation in resting CLL cells during CsA therapy. Furthermore, expression of CD25 and CD69 by activated CD4 T cells and expression of CD38 and CD69 by activated CLL cells was reduced in this case. Counter-intuitively, there appeared to be an increase in the proliferation rate of CLL cells and an overall small reduction in the total lymphocyte count following commencement of CsA. One explanation for these findings is that there may have been a component of anergy that was reversed in the presence of CsA, leading to an increased rate of apoptosis of CLL cells. To investigate this further, signalling-response to BCR-ligation should have been measured before and during CsA therapy.

PD-1 expression by CD4 T cells has been reported to be CsA- sensitive, and as a key component in immune evasion in CLL, was an important line of investigation on this study. However, we found that PD-1 expression was not reduced after 4 weeks CsA therapy. Furthermore, T cell phenotype remained remarkably stable post CsA, with preservation of expanded CD8 effector memory and effector compartments.

Comparison of gene expression on CLL and CD4 T cells pre and post *in-vivo* CsA therapy was performed to further investigate the effect of CsA on CD4 T cell: CLL cell interactions *in-vivo*. Comparison of CD4 T cell gene expression pre and post treatment was only possible in three patients due to insufficient mRNA from the fourth patient. However, amongst the three genes down-regulated on treatment, was *PTGER4*, which encodes prostaglandin E receptor 4 (EP4). This g-protein coupled receptor is reported to transmit pro-inflammatory signalling, leading to Th1/Th17 skewing *in-vivo*. Although not previously reported to contribute to the mechanism of action of CsA, down-regulation is consistent with *in-vitro* and *in-vivo* effects of the drug.

There are a number of limitations to this study; the major challenge being recruitment. Firstly, the trial protocol defined 'poor risk' as expression of CD38 in > 7% CLL cells. Many of the patients found to meet this criterion had evidence of disease progression that warranted conventional therapy at the time of diagnosis. Secondly, the trial protocol involved 24 weeks of continuous patient commitment with a minimum of weekly hospital visits during the eight weeks of CsA therapy. This degree of commitment was prohibitive for most patients approached regarding the study, meaning that only four patients (of fourteen approached) were recruited in the initial phase. Following a protocol amendment to reduce the protocol duration to 16 weeks, one further patient was recruited. Despite major efforts to recruit patients, including an attempt to open the study at Southampton General Hospital, the small number of patients recruited meant that this study lacked statistical significance.

Secondly, there is a large pool of non-proliferating cells in patients with CLL leading to 'dilution' of the activated/ proliferating sub-population. Measurement of proliferation in the lymph-node compartment may have been a valuable method of targeting proliferating CLL cells, but serial lymph-node sampling was beyond the scope of this investigation.

Finally, four weeks was a relatively short duration of exposure to CsA. In view of the kinetics of proliferation, release and disappearance of cells explored in detail in Chapter 5, a longer treatment interval before measuring the impact of CsA on proliferation may have been more informative.

If this investigation were to be repeated, simplification of the research protocol would be the greatest priority. The protocol would involve baseline labelling with deuterated glucose before commencement of CsA after two weeks. Following eight weeks of CsA therapy, labelling would be performed for a second time. Serial blood samples would be taken for two weeks after the second labelling, doubling the exposure time to CsA but reducing patient commitment to twelve weeks.

In summary, despite the promising *in-vitro* effect of CsA in CLL, the CyCLLe study did not demonstrate a significant biological or clinical effect of CsA in patients with early stage, adverse-risk disease. It does however highlight the importance of clinical-trial design in both answering clinical questions whilst ensuring that the degree of patient commitment is acceptable.

Chapter 5 . *In-Vivo* Studies of Tumour Kinetics in CLL

5.1 Introduction

Chronic lymphocytic leukaemia is characterised by considerable clinical heterogeneity that reflects major differences in the capacity of tumour cells to proliferate. It is widely accepted that CLL cells are dependent upon signalling from the tumour microenvironment for survival and proliferation. Furthermore, there is robust evidence for the role of BCR signalling in driving the proliferation of CLL cells within the tissue compartment. In a comparative gene expression study of lymph-node and peripheral blood derived CLL cells, Herishanu *et al* demonstrated that BCR signalling is the most prominent pathway up-regulated in lymph-node resident CLL cells, and that proliferation measured by Ki67 was four-fold greater in the LN compared to the PB (Herishanu *et al.*, 2011). Detailed characterisation of the relationship between migration of tumour cells into the tissue-compartment, BCR signalling and proliferation is fundamental to understanding the disease biology that underpins the clinical heterogeneity in CLL.

A major contributing factor to whether patients experience early disease progression is the *IgV_H* mutational status. On a clinical level, patients with unmutated *IgV_H* genes (UM-CLL) demonstrate a short time to first treatment and reduced progression-free and overall survival (Damle *et al.*, 1999; Hamblin *et al.*, 1999). *In-vitro* studies have elegantly demonstrated that in UM-CLL, there is increased sIgM expression and enhanced calcium-flux, SYK phosphorylation and proliferation in response to BCR ligation (Guarini *et al.*, 2008; Mockridge *et al.*, 2007). In contrast, in patients with mutated *IgV_H* genes (M-CLL), CLL cells are programmed towards 'anergy' ie a state of unresponsiveness to sIgM stimulation, characterised by constitutive activation of ERK and NFAT (Muzio *et al.*, 2008). In health, anergy acts to silence auto-reactive B lymphocytes following low-affinity antigen binding, leading to their elimination by apoptosis (Goodnow, 1997). However, CLL cells are protected from apoptosis, permitting the survival of anergic cells.

The distinction between UM-CLL and M-CLL is not absolute, Apollonio *et al* did not demonstrate a statistically significant relationship between IgM-induced ERK

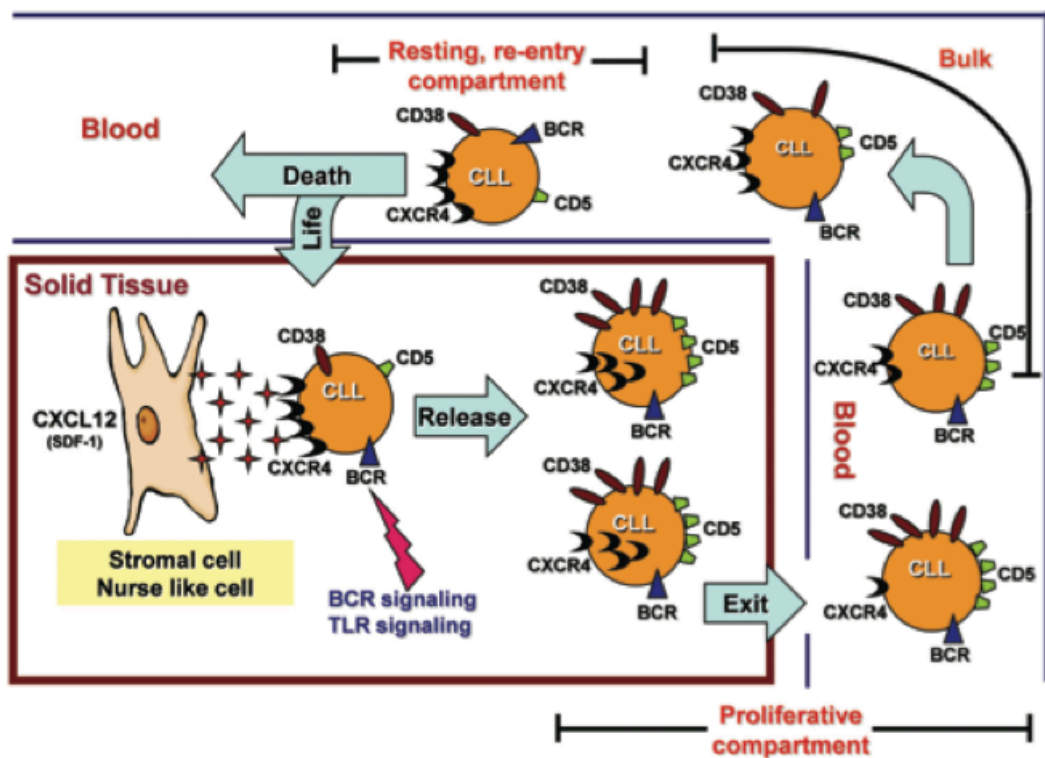
phosphorylation and *IgV_H* mutational status (Apollonio et al., 2013). An important discriminator in patients with M-CLL is the degree of sIgM expression, with high expression significantly associated with ability to signal through the BCR (Mockridge et al., 2007). Furthermore, there is heterogeneous signalling within the cohort of M-CLL patients in which there is uniform sIgM expression, suggesting that there are additional influences on BCR signalling that are likely to be environmental (Apollonio et al., 2013).

The clinical outcomes of patients with M-CLL are highly variable. Expression of the cell surface molecules CD49d and CXCR4 that are functionally related to the migratory potential of tumour cells has recently been shown to be predictive of overall survival within this cohort of patients (Pepper et al., 2015). High expression of CXCR4 and CD49d identified a cohort of patients with a prognosis similar to that of patients with UM-CLL, further emphasising the importance of migration in the pathogenesis of CLL.

In view of the role of the tumour microenvironment in the pathogenesis of CLL, characterisation of trafficking of proliferating CLL cells between peripheral blood and tissue compartments is an important line of investigation. Calissano et al investigated this relationship using *in-vivo* labelling with deuterated water; all proliferating CLL cells incorporate deuterium into DNA and 'enrichment' can be measured by GC-MS. They went on to sort deuterium labelled peripheral blood CLL cells according to co-expression of CD5 (a marker of recent BCR signalling) and the chemokine receptor CXCR4 (required for tissue-migration via binding with SDF-1), taking the 10 % of cells at each extreme and the intermediate 10 % cells. They found that the sub-population with the lowest CXCR4 and highest CD5 (CXCR4^{low}/ CD5^{hi}) expression had 11 x greater deuterium enrichment than the subpopulation with the reciprocal phenotype, demonstrating that this represented the most recently proliferated sub-population. Global gene expression studies found that CXCR4^{low}/ CD5^{hi} cells expressed pro-proliferation and anti-apoptosis genes, whereas CXCR4^{hi}/ CD5^{low} cells preferentially expressed anti-proliferative genes and genes associated with lymph-node homing e.g *CCR7* (Calissano & Damle, 2011). They went on to propose that there is a continuum between proliferating and resting sub-populations, whereby following migration and proliferation in the lymph-node, CXCR4 is down-regulated and cells are released into the peripheral blood. Recently proliferated CLL cells subsequently enter a resting state,

characterised by loss of CD5 and re-expression of CXCR4, after which they either undergo apoptosis or re-enter the lymph-node to undergo a further round of proliferation.

This model is supported by *in-vitro* studies that have utilised a circulation system in CLL; it was reported that circulating CLL cells re-express CXCR4 and that following migration across an endothelial layer, CXCR4 is down-regulated (Pasikowska et al., 2016; Walsby et al., 2014). Additionally, it has been reported that ligation of the BCR leads to a phenotypic change that includes down-regulation of CXCR4 (Quiroga et al., 2009), leading to dissociation from SDF-1 expressed by stromal cells. However, lymph-node re-entry by circulating CLL cells has not been demonstrated *in-vivo*.



(Calissano & Damle, 2011)

Figure 5.1 Hypothetical Model of CLL Trafficking

This model of trafficking illustrates the hypothesis that CLL cells undergo proliferation in the lymph-node compartment. Proliferating cells characterised by high CD5/ high CD38 and low CXCR4 expression are then released into the peripheral blood compartment where CXCR4 is re-expressed and CD5 is down regulated. Recently proliferated cells subsequently undergo apoptosis or re-enter the lymph-node compartment to under-go a further round of proliferation.

In view of the sub-clonal heterogeneity observed by Calissano et al and in light of the major role that BCR signalling plays in CLL proliferation, Coelho et al went on to investigate the intra-clonal relationship between sIgM expression and phenotypic and functional characteristics including CXCR4 expression, BCR signalling capacity and proliferation. CLL sub-groups were defined by the degree of sIgM expression (based on bead-bound anti-IgM (sub-groups 1-4: subgroup 1 with the lowest degree of bead binding to subgroup 4 with the highest bead binding visualised by image flow cytometry)). They found that highest proportion of cells was in subgroup 1, which was enriched with cells expressing the activation markers CD25 and CD38 and cells that had recently proliferated (Ki67 positive). Cells in the highest sub-group expressed high CD5, high CXCR4 and had the greatest increase in pERK upon sIgM ligation. It was concluded that the subgroup 1 represented a post-proliferative fraction whilst subgroup 4 represented a potentially dangerous cohort of cells, primed for lymph-node entry, BCR signalling and proliferation (Coelho et al., 2013).

These findings are at odds with the *in-vivo* labelling studies that report that the recently proliferated fraction is characterised by CXCR4^{low}/CD5^{hi}. This may be due to the fact that recently proliferated CLL cells have undergone complex and dynamic phenotypic changes that may be poorly represented in static *in-vitro* systems.

In-vivo labelling studies provide the most accurate representation of tumour kinetics but are challenging to perform in an observational setting where long-term patient commitment is essential. *In-vivo* labelling of proliferating cells can be achieved using deuterium, a non-radioactive isotope of hydrogen, delivered either as deuterated water or deuterated glucose. Deuterated water is more suitable for labelling slowly proliferating cells; as the body water pool is large, daily administration of 60ml of deuterated water is required to achieve 1-5 % labelling and must be maintained for 84 days to study proliferation (Messmer et al., 2005). The wash-out period is also slow (104 days), thus this method isn't suitable for sequential labelling e.g. to investigate the effect of a drug on proliferation. In view of the slow on-set /off-set of labelling, this method is also unsuitable for labelling populations of cells that rapidly proliferate or disappear (due to apoptosis or change in phenotype). In comparison, deuterated glucose is more appropriate for labelling rapidly proliferating and transient populations. The body glucose pool is small (15g), with a half-life of two hours allowing

rapid achievement of labelling (40 % deuterium enrichment) that can be maintained by regular oral administration of 10ml of deuterated glucose every 30 minutes. Rapid disappearance of deuterated glucose on cessation of intake produces a clearly defined pulse of labelled cells that can be tracked over time in the peripheral blood and tissue compartments.

Orally administered deuterated glucose has previously been used to compare proliferation rates of CLL cells with healthy B lymphocytes. Seven patients with CLL and seven healthy controls were studied; deuterium enrichment was measured in purified populations from peripheral blood samples that were drawn from 3-21 days post labelling. The proliferation rate of CLL cells was lower than that of healthy B lymphocytes (0.47 %/d vs 1.31 %/day $p=0.007$) but combined with a reduced disappearance rate, appeared to result in reduced cell turnover and net accumulation of cells (Defoiche et al., 2008). The mean proliferation rate in this small study was remarkably similar to that reported from the deuterated water studies (0.44 %/day) (Messmer et al., 2005) and there was considerable heterogeneity between patients.

We sought to investigate *in-vivo* tumour kinetics in CLL using orally delivered deuterated glucose in a cohort of ten patients with non-progressive disease.

We started by measuring the proliferation rate of the entire clone of CLL cells in each individual and then went on to assess intra-patient variation in proliferation by repeating deuterium labelling after eight weeks. The degree of spontaneous variation in proliferation is important to assess whether '*in-vivo* proliferation' can be used to measure the effect of an intervention in clinical trials.

The relationship between clonal proliferation rates and phenotypic and functional parameters associated with proliferation was then investigated by measuring CD38 expression and BCR-ligation induced ERK phosphorylation. Finally, clonal CLL proliferation rates were correlated with telomere length to investigate if point proliferation rates were reflective of the tumour's replicative history. Telomere length has previously been shown to be a highly predictive prognostic marker in CLL, with shortened telomeres being predictive of shortened time to first treatment and inferior overall survival (T. T. Lin et al., 2010; Ricca et al., 2007; Rossi et al., 2009).

We then went on to investigate trafficking of CLL cells between the lymph-node compartment and the peripheral blood. The kinetics of release and disappearance of recently proliferated CLL cells were previously studied in 1960s using intravenous tritiated thymidine, followed by measurement of radioactivity in leukocyte smears (Zimmerman, Godwin, & Perry, 1968). These studies reported that there were two patterns of labelling, the first pattern indicating a small population of rapidly proliferating cells that was relatively short lived. The second pattern, observed in patients with splenomegaly, indicated a population of long-lived cells that were detectable at eight weeks after labelling.

We aimed to accurately establish rates of release and disappearance of recently proliferated CLL cells in the peripheral blood compartment by sampling peripheral blood at regular intervals over an eight-week period. There are a number of factors that contribute to tissue homing and retention including CXCR4 and CCR7 expression, whilst egress is determined by expression of the sphingosine-1-phosphate receptor (S1P1). It has previously been reported that in CLL, defective S1P1 expression contributes to extended survival by prolonging the residency of the tumour cells in the pro-survival niche (Capitani et al., 2012).

Disappearance of CLL cells from the circulation can occur due to cell death, homing to tissue-sites or by a change in phenotype. Disappearance rates of recently proliferated CLL cells have previously been investigated by Defoiche et al who measured deuterium enrichment in CLL cells for up to 21 days and concluded that rates of disappearance were reduced compared to healthy B lymphocytes. We went on to measure enrichment for a minimum of 56 days and for 84 days in a sub-set of patients to fully characterise the kinetics of disappearance of CLL cells in our cohort of patients.

As a sub-study to further investigate trafficking between the peripheral blood and lymph-node compartments, we went on to investigate if a proportion of labelled CLL cells re-enter the lymph-node. Although *in-vitro* studies have shown that the chemokine receptor CXCR4 is re-expressed in circulating CLL cells (Walsby et al., 2014), and that this is thought to enable lymph-node re-entry, this has not been demonstrated *in-vivo*. Establishment of tissue homing *in-vivo* is fundamental to the characterisation of CLL pathogenesis.

We further investigated the relationship between trafficking and proliferation using the strategy described by Calissano et al, measuring deuterium enrichment in peripheral blood sub-populations defined by CXCR4 and CD5 expression. In doing this, we took advantage of the 'pulse' labelling, achievable only with deuterated glucose labelling, to further investigate changes in labelling in the sub-populations over an extended period of time.

The sub-populations described by Calissano et al have only been demonstrated in peripheral blood CLL cells, with the hypothesis that following release from the lymph-node, there is dynamic expression of CXCR4 and CD5 in circulating CLL cells that enables lymph-node re-entry. We therefore measured deuterium enrichment in lymph-node derived CLL cells according to expression of CXCR4 and CD5 to assess if sub-populations with distinct proliferative characteristics exist outside the peripheral blood compartment.

In view of the role that BCR signalling plays in driving CLL proliferation, we sought to investigate the relationship between BCR expression, function, trafficking and proliferation. It has been reported that sIgM expression and BCR signalling is up-regulated in lymph-node derived CLL cells (Coulter et al (manuscript submitted)) (Herishanu et al., 2011). Furthermore, it has recently been reported that IL4, possibly derived from CLL T cells, augments CD79b and sIgM expression and BCR signalling in the CLL lymph-node (Aguilar-Hernandez et al., 2016; Guo et al., 2016). However, it is unclear what happens to BCR expression in the wake of proliferation. One hypothesis is that the BCR is internalised upon antigen engagement and that re-expression occurs slowly. *In-vitro* studies have shown that maximum recovery of sIgM expression and signalling capacity occurs after 48-72 hours' incubation (Mockridge et al., 2007). This would suggest a model where the BCR is internalised following antigen engagement in the lymph-node and is slowly re-expressed following release into the peripheral blood compartment. It is clear that the microenvironment plays a key role in BCR expression in CLL and as such, investigations into the dynamics of BCR expression are best-performed *in-vivo*. We therefore measured deuterium enrichment in CLL sub-populations according to sIgM expression, taking the highest, intermediate and lowest decade of cells.

The relationship between sIgM expression, BCR internalisation and BCR signalling-capacity has recently been investigated by Coulter et al (manuscript submitted). It was found that although CLL cells express low levels of sIgM, the capacity to internalise the BCR is increased in leukaemic cells and that sIgM expression is the principal factor that affects internalisation. They found that lymph-node derived CLL cells exhibited increased sIgM expression and BCR internalisation compared to peripheral blood derived CLL cells and that capacity to internalise the BCR positively correlated with CD5 expression.

To further investigate the relationship between BCR expression, BCR internalisation and proliferation, we went on to measure deuterium enrichment in subpopulations defined high, intermediate and low BCR internalisation, using a pH sensitive dye linked to agonistic IgM.

In summary, we performed a detailed investigation into the kinetics of proliferation and trafficking between peripheral blood and lymph-node compartments using the novel approach *in-vivo* labelling with deuterated glucose. We sought to characterise the relationship between BCR expression, internalisation, proliferation and trafficking between the tumour microenvironment and peripheral blood in a small cohort of patients with non-progressive CLL. This investigation is particularly relevant in an era where we are increasingly using drugs that inhibit BCR signalling and that have consequential effects on proliferation and trafficking to treat patients with symptomatic CLL. Improved characterisation of the underlying pathophysiology will enable us to better understand the heterogeneous effects of BCR antagonists *in-vivo*.

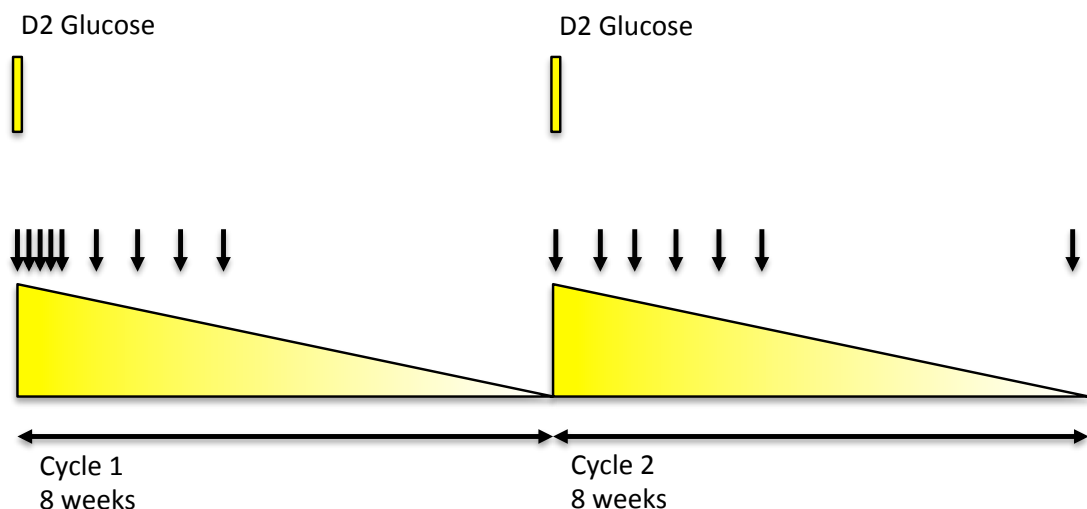
5.2 Aims and Objectives

1. To determine the proliferation rate of CLL cells in a cohort of patients (n=10) with non-progressive disease using *in-vivo* labelling with deuterated glucose and to determine the degree of intra-patient variation in proliferation rate over a eight week period.
2. To investigate the relationship between point-proliferation rate and CLL telomere length in a cohort of patients with non-progressive disease.
3. To investigate the relationship between phenotypic and functional characteristics and CLL proliferation rate.
4. To investigate the rate of release of proliferated CLL cells from the lymph-node compartment into the peripheral blood compartment and the subsequent disappearance rate of proliferated cells from the peripheral blood.
5. To investigate the relationship between BCR expression, BCR function (internalisation), proliferation and trafficking using deuterium labelling to track proliferating CLL cells.

5.3 Materials and Methods

5.3.1 Trial Design

A single-centre, observational cohort study was designed to investigate tumour kinetics in ten patients with non-progressive Binet Stage A/B CLL who had not received treatment in the previous three months. The study consisted of two cycles whereby labelling with deuterated glucose was performed on Day 0 of each cycle. Release rates were investigated by performing daily blood tests for the first week of Cycle 1, following which blood was drawn weekly for the subsequent four weeks and after eight weeks to investigate disappearance of labelled cells (Figure 5.2). Intra-patient variation in proliferation was measured by comparing proliferation rates from Cycles 1 and 2. Patients with palpable lymph-nodes were offered the opportunity to participate in a sub-study where lymph-node sampling was performed to further investigate trafficking between the peripheral blood and lymph-node compartments.



Primary End-points:- i. Rate of release of recently proliferated CLL cells
ii. Correlation of CLL proliferation rate with telomere length
iii. Intra-patient variation in tumour proliferation

Figure 5.2 Schema for *In-Vivo* Studies of Tumour Kinetics In CLL

Deuterated glucose labelling was performed in two occasions, separated by 8 weeks. In Cycle 1, blood sampling took place daily for the first week, weekly for the first month and then after two months. In Cycle 2, blood sampling took place on days 4, 7, 14, 21, 28 and 56.

5.3.2 Eligibility

Patients with non-progressive Binet Stage A or B CLL who had not have received therapy for at least 3 months and had no current indication for therapy, were eligible. Exclusion criteria included diabetes mellitus (due to the risk of deranged glycaemic control on labelling days), active autoimmune disease requiring immunosuppressive therapy, pregnancy/lactation, positive serology for HIV, Hepatitis C or active infection with Hepatitis B and other active malignancy.

Patients were identified by clinicians working within the CLL service at King's College Hospital (Denmark Hill and Princess Royal University Hospital Sites) and were given a Patient Information Sheet to consider for a minimum of 24 hours before the consent visit. The patient provided written, informed consent before undergoing screening investigations. Consent and screening was performed by Dr. Cuthill.

5.3.3 Deuterium Labelling

Labelling was performed as described for the CyCLLe Study (described in 4.2.2). All labelling was performed by Dr Cuthill with the support of trained Clinical Trials Nurses in the Clinical Trials Facility at King's College Hospital.

5.3.4 Blood Sampling

6ml peripheral blood was drawn in Lithium heparin tubes at the specified time points.

5.3.5 PBMC isolation and storage

Peripheral blood mononuclear cells were immediately isolated using Ficoll Hypacque Density Centrifugation as described in 2.2. PBMCs were rapidly frozen as described in 2.5. Four aliquots of PBMCs were stored at each time-point for each patient.

5.3.6 Lymph-node Sampling

Patients who agreed to participate in this sub-study (Ethics) provided written consent at the time of each fine- needle aspirate. The skin was cleaned with chlorhexidine impregnated sponge and allowed to dry. A 12 gauge needle was passed into the lymph-node and tissue was multiply aspirated into a 10 ml syringe. Suction was released before withdrawing the needle. The tissue was expelled into a specimen container containing 2ml RPMI.

The specimen was transferred to the laboratory and was centrifuged at 300 *g* for 5 minutes. The supernatant was discarded and cells re-suspended in 2 ml red cell lysis buffer at 37°C for 15 minutes. The buffer was then removed by centrifugation at 300 *g* for 5 minutes and cells were washed x 2 in PBS/ 5mM EDTA and incubated with CD5 (FITC), CD19 (APC) and CXCR4 (PE). Cells were washed x 2 in PBS/ 10mM EDTA and passed through a nylon mesh to remove clumps. DAPI (1:1000) was added 5 minutes before flow sorting. Cells were flow-sorted using the BD FACS Aria™ II Cell Sorter at the core flow facility, Biomedical Research Centre, Guy's Hospital. Cells were collected in 1.5 ml Eppendorf tubes and were stored as dry pellets in a monitored -80°C freezer before they were transported to St George's Hospital on dry ice.

5.3.7 PBMC recovery and fluorescence activated cell sorting

PBMCs from all time-points within a cycle were recovered simultaneously, as described in 2.6. Cells were rested at 37°C in RPMI for one hour before labelling with antibodies for cell sorting. Labelling was performed on ice and CLL cells were washed twice with PBS. DAPI was used to label non-viable CLL cells.

5.3.8 CXCR4/ CD5 sub-population sorting

10^7 PBMCs were rapidly thawed and recovered at 37°C in RPMI for 60 minutes. They were then washed with PBS and labelled with CD5 (FITC), CD19 (APC) and CXCR4 (PE). Antibodies were incubated with cells on ice for 15 minutes before being washed twice with ice-cold PBS. Cells were re-suspended in 2 ml PBS containing 2 % FBS and 5mM EDTA. 1mcl DAPI (1:100) was added 5 minutes before acquisition. Cells were flow-sorted using the BD FACSaria™ II Cell Sorter as above. The gating strategy is illustrated below. Three sub-populations of CLL cells were collected, defined by CXCR4hi/ CD5low, CXCR4int/CD5int and CXCR4low/ CD5 hi. 5×10^5 - 1×10^6 cells from each population were collected in 1.5ml Eppendorf tubes. Dry pellets were made and stored at -80 °C before they were transported to St George's Hospital on dry ice.

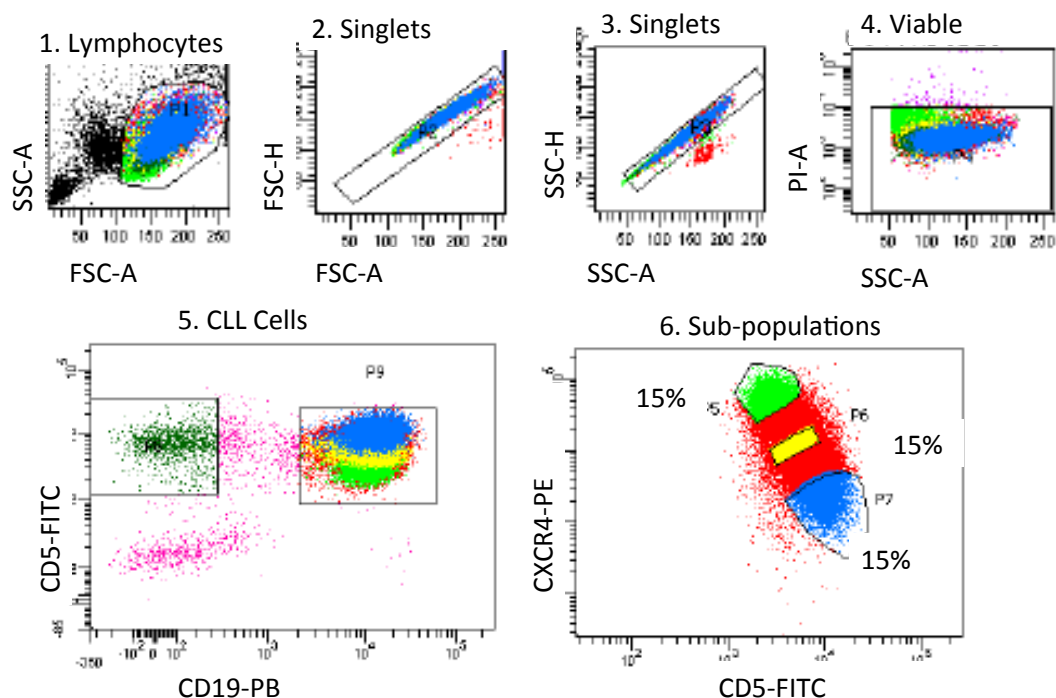


Figure 5.3 Gating strategy for FACS sorting of CLL cells and sub-populations

Single, viable CLL cells (CD5+/CD19+) were FACS sorted into cohorts according to expression of CXCR4 and CD5, the highest, intermediate and lowest 15 % cells. A minimum of 100,000 cells was collected from each cohort.

5.3.9 Surface IgM Sorting

10^7 PBMCs were rapidly thawed and recovered at 37°C in RPMI for 60 minutes. Cells were washed and labelled with CD5 (PECy7) and CD19 (PB). Antibodies were incubated with cells at 4°C for 15 minutes before being washed twice with PBS. Immediately before sorting, cells were incubated with anti-IgM (FITC) on ice for 15 minutes. Cells were washed twice with ice-cold PBS containing 2 % FBS and 5mM EDTA. 1mcl DAPI (1:100) was added 5 minutes before acquisition and was incubated on ice. Cells were flow-sorted using the BD FACSARIA™ II Cell Sorter. The gating strategy is illustrated below (Figure 5.4). Three sub-populations of CLL cells were collected, defined by 1) IgM low (lowest 10 %), 2) IgM intermediate (middle 10 %) and IgM high (top 10 %). 5×10^5 - 1×10^6 cells from each population were collected in 1.5ml Eppendorf tubes. Dry pellets were made and stored at -80°C before transporting to St George's Hospital on dry ice.

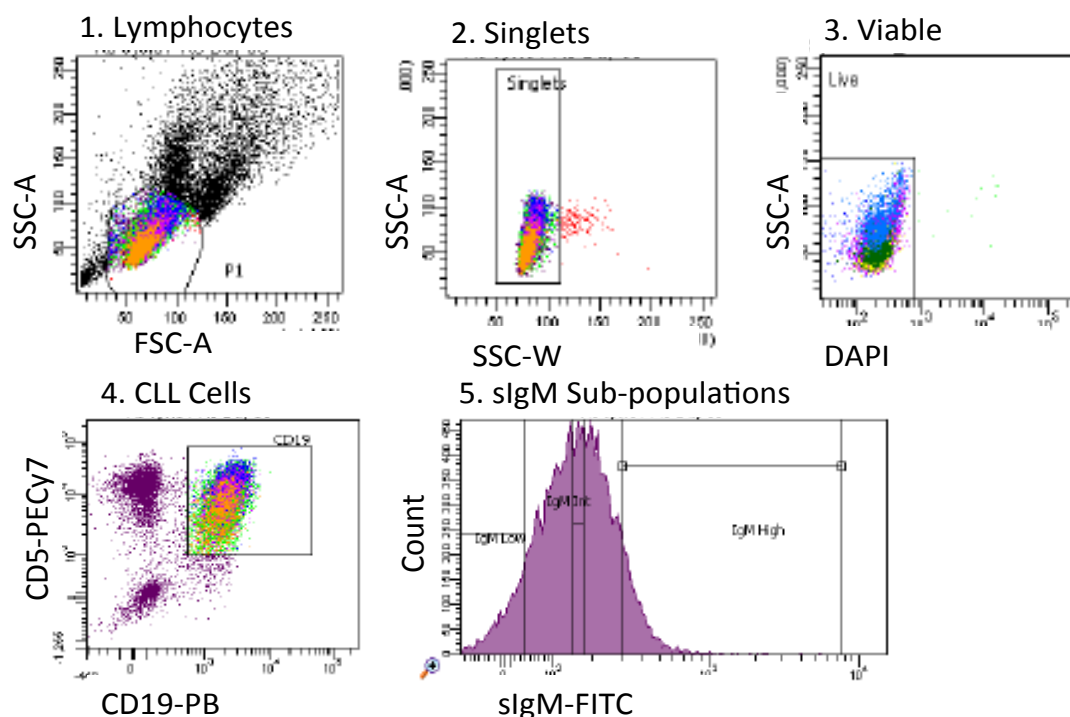


Figure 5.4. Gating Strategy for sIgM FACS sorting

Aliquots of cryopreserved CLL cells were recovered and labelled with FITC-conjugated anti- sIgM antibody. Single, viable CLL cells expressing low, intermediate and high levels of sIgM were flow-cytometrically sorted.

5.3.10 BCR Internalisation Assay

BCR internalisation assays were performed by Dr Eve Coulter using a locally developed assay (Dr Eve Coulter, manuscript submitted) that use a pH sensitive dye (pHrodo™ Red avidin (Life Technologies, Paisley, UK)) conjugated to anti-IgM. Outside the cell, where pH is neutral, there is no fluorescence whereas within the cell, pH = 5 and the dye fluoresces. Therefore, following BCR ligation and receptor internalisation, a fluorescent signal is emitted and is measurable by flow cytometry (Figure 5.5).

Briefly, one microgram of pHrodo™ Red Avidin was incubated with or without an equimolar amount of biotinylated goat F(ab')₂ anti-human Immunoglobulin M (αIgM; Cambridge Biosciences, Cambridge, UK) for one hour at room temperature to allow formation of labelled complexes. Target cells (5x10⁵) were incubated with pHrodo-avidin-αIgM (pHrodo-αIgM) for 30 minutes at 4°C to allow initial receptor binding, then at 37°C for a further one hour to allow for internalisation. All incubations were performed in PBS pH 7.4, so that any observed fluorescence was due to BCR internalisation and trafficking of the pHrodo-αIgM to acidified endosomes. BCR internalisation was expressed as the percentage of cells with fluorescence above that of the pHrodo-avidin negative control.

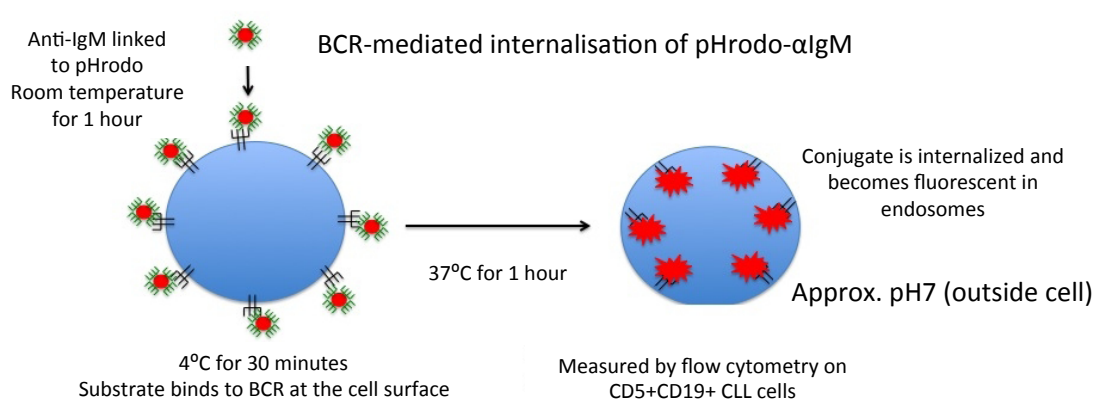


Figure 5.5 pHrodo™ application to measure BCR internalisation

pHrodo is a fluorogenic dye that increases in fluorescence as the pH of its surrounding falls. The dye was conjugated to anti-sIgM to assess BCR internalisation in CLL cells via flow cytometry.

5.3.11 BCR Internalisation Sorting

10^7 PBMCs were rapidly thawed and recovered at 37°C in RPMI for 60 minutes. PBMCs were washed with PBS and incubated with 100mcl of the pHrodo cocktail at 4°C for 30 minutes. PBMCs+ cocktail were then transferred to a 37°C incubator for 60 minutes. PBMCs were washed in ice- cold PBS and were surface-stained with CD5 (APC) and CD19 (PB) on ice for 15 minutes. PBMCs were washed twice in PBS. DAPI was incubated with the cells or five minutes on ice immediately before sorting. The gating strategy below was used; we collected the top, middle and lowest 10 % viable CLL cells according to ability to internalise the BCR. Gates were manipulated during the sort to maintain 10 % cells in each gate.

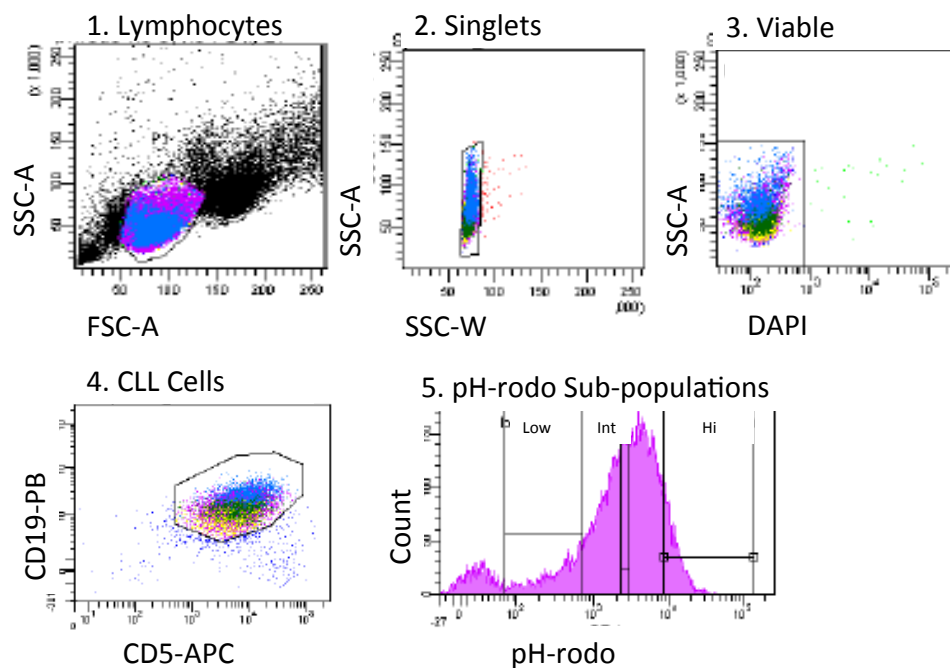


Figure 5.6 Gating Strategy for BCR internalisation FACS sorting

Aliquots of cryopreserved CLL cells were recovered, labelled with avidin- α IgM (pHrodo- α IgM) and incubated to allow internalisation. Single, viable CLL cells with low, intermediate and high levels of fluorescence indicated BCR internalisation were flow-cytometrically sorted.

5.3.12 Gas Chromatography Mass Spectrometry

Gas chromatography mass spectrometry (GC MS) was performed by Dr. Yan Zhang in Prof. Macallan's laboratory at St. George's Hospital, as per the method detailed in 4.2.8.

5.3.13 Single Telomere Length Analysis

Telomere length was analysed by our collaborator, Prof. Baird, at Cardiff University. DNA was extracted, solubilized, quantified and diluted. Multiple polymerase chain reactions (PCRs; typically 6 reactions per sample) were carried out for each test DNA with telomere-adjacent and Teltail primers. The reactions were cycled with an MJ PTC-225 thermocycler. The DNA fragments were resolved by 0.5 % Tris acetate ethylenediaminetetraacetic acid agarose gel electrophoresis, and detected by 2 separate Southern blot hybridizations with random-primed α -³³P– labeled TTAGGG repeat probe and a telomere-adjacent probe, together with a probe to detect the 1-kb and 2.5-kb molecular weight markers. The hybridized fragments were detected by phosphorimaging with a Molecular Dynamics Storm 860 phosphorimager. The molecular weights of the DNA fragments were calculated using the Phoretix 1D quantifier.

5.3.14 Trial Administration

The study was sponsored by King's College Hospital NHS Foundation Trust and was included in the National Institute for Health Research Clinical Research Network (NIHR CRN) portfolio. The trial received peer-reviewed funding from Leukaemia and Lymphoma Research (now called 'Bloodwise').

Ethical approval was granted by the National Research Ethics Committee (13/LO/0434).

Two substantial amendments were approved. Amendment 1 (August 2013) removed the requirement for patients to express > 7 % CD38 from the inclusion criteria. This was because the initial inclusion criteria were deemed to be too restrictive and a barrier to recruitment. Amendment 2 (April 2015) was submitted to enable the recruitment for six further patients with unmutated *IgV_H* genes. This protocol was amended to a single cycle (one labelling day) with extended peripheral blood sampling, up to 12 weeks.

Dr Cuthill performed all trial administration including application through the Integrated Research Application System, amendment submission and maintenance of recruitment data for the NIHR CRN portfolio. The study was reviewed monthly at the King's College Hospital CLL Clinical Trials Meeting.

5.4 Results

5.4.1 Patient Characteristics

Ten patients were recruited to the study (Table 5.1). By chance, all patients were found to have mutated *IgV_H* genes (M-CLL). A study amendment has been submitted to enable the recruitment of a further cohort of patients with unmutated *IgV_H* genes (UM-CLL). Notably, three patients expressed CD38 at a level of $\geq 7\%$.

Table 5.1 Characteristics of patients recruited to the *In-Vivo* Kinetics Study.

Trial Code	Age (yrs)	Sex	Lymphocyte Count ($\times 10^9/\text{dl}$)	Binet Stage	Previous Rx	<i>IgV_H</i> (M/UM)	CD 38	Cyto-genetics
K1	66	F	7	A	Nil	M	7 %	Normal
K2	55	F	200	B	Nil	M	0 %	13q-
K3	38	F	20	A	Nil	M	1 %	Normal
K4	67	F	60	A	Nil	M	0 %	Normal
K5	62	M	30	A	Nil	M	0 %	Normal
K6	68	F	200	B	Nil	M	3 %	13q-
K7	65	M	90	B	Nil	M	0 %	13q-
K8	69	M	39	B	Nil	M	12 %	Trisomy 12
K9	54	M	100	B	Nil	M	0 %	Normal
K10	53	M	20	A	Nil	M	51 %	Trisomy 12

5.4.2 Assessment of proliferation rate and of intra-patient variation in peripheral blood CLL cells

The proliferation rate of CLL cells was calculated by dividing the maximum deuterium enrichment in CLL cell DNA by the plasma glucose deuterium enrichment corrected for intracellular dilution. Proliferation rates were heterogeneous and varied from 0.08 %/day to 0.95 %/day. The mean proliferation rate in our population of patients with non-progressive M-CLL was 0.36 %/day. As expected, this is lower than the mean proliferation rate of 0.44 %/day reported by Messmer et al in a cohort of 18 patients, half of whom had UM-CLL and half of whom had progressive disease (Messmer et al., 2005).

We sought to investigate the degree of spontaneous intra-patient variation in proliferation by repeating *in-vivo* labelling with deuterated glucose after eight weeks. Measurement of spontaneous variation in proliferation is essential if this method is to be applied to assess the effect of an intervention on proliferation.

There was significant positive correlation between cycles 1 and 2 ($r=0.6331$ $p=0.049$), as illustrated in Figure 5.7. In one patient, there was a doubling of proliferation between cycles 1 and 2 in the absence of overt clinical features.

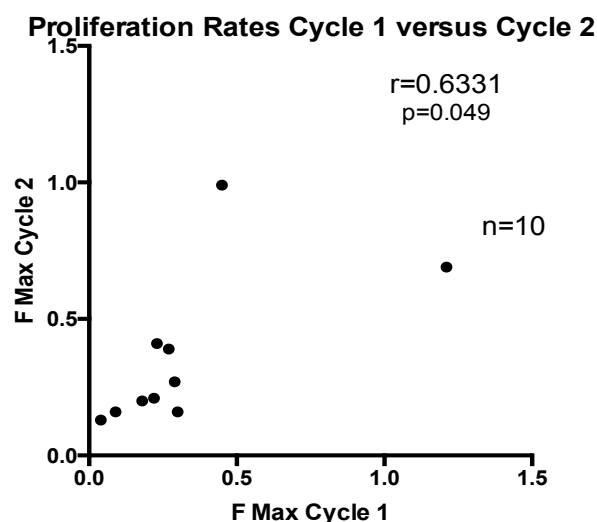


Figure 5.7 Correlation between proliferation rates of peripheral blood CLL cells

Proliferation rates were measured on two occasions, spaced by eight-weeks, in ten patients. Proliferation rate was calculated using maximum deuterium enrichment in purified peripheral blood CLL cells.

5.4.3 Relationship between sIgM expression, BCR signalling, CD38 expression and proliferation

The proliferation of CLL cells is thought to result, at least in part, from antigen ligation and signalling through the BCR. However, BCR responses are heterogeneous both between patients and within a patient. In patients with UM-CLL, sIgM expression and BCR signalling responses are stronger and are associated with proliferation whereas in patients with M-CLL, sIgM expression is lower, there is a lack of BCR signalling and these cells are programmed towards anergy (Apollonio et al., 2013; Guarini et al., 2008; Mockridge et al., 2007). However, within the M-CLL group, there is considerable heterogeneity; some patients have high sIgM expression, similar to the UM-CLL group, and these patients signal through the BCR.

The proliferation of CLL cells has previously been linked to the expression of CD38, a molecule that serves both as an ectoenzyme and receptor (Deaglio et al., 2003; Morabito et al., 2006). Within a CLL clone, it has been shown that the CD38⁺ fraction is enriched for recently proliferated cells (Calissano et al., 2009; Damle et al., 2007). CD38 expression is upregulated in the lymph-node compared to the peripheral blood in a process that has been shown to be dependent on signals from the tumour microenvironment, specifically activated CD4 T cells (O. Jaksic et al., 2004; Patten, Buggins, Richards, Wotherspoon, Salisbury, Mufti, Hamblin, & Devereux, 2008b; Willimott et al., 2007). Investigations into the impact of CD38 expression on BCR signalling have yielded conflicting results; most recently, it has been shown that in patients with UM-CLL, 'signallers' generally expressed CD38 whilst there this relationship did not hold for patients with M-CLL (Lanham et al., 2003; Mockridge et al., 2007; Zupo et al., 1996). This reinforces the hypothesis that CD38 expression influences CLL activation and proliferation through mechanisms that are independent of the BCR.

To investigate the association between sIgM expression, CD38 expression, BCR signalling and proliferation in our cohort of patients with M-CLL, we measured the phenotypic and functional parameters in relation to *in-vivo* proliferation rates (Table 5.2). The expression of sIgM and CD38 was measured by flow cytometry (Median fluorescent intensity (MFI) and percentage expression, respectively), and pERK

activation in response to agonistic sIgM ligation was assessed by Western Blotting (Figure 5.8). Total and phospho-ERK was measured before and after sIgM stimulation, using Western Blotting (Chapter 2.6). Signal strength was quantified using densitometric analysis (Image J Software). Activation ratio was calculated by dividing the ratio of stimulated pERK by the ratio of stimulated ERK. In patients K2 and K9, there was activation of ERK phosphorylation following IgM stimulation.

We did not observe a direct association between sIgM expression and proliferation in our small cohort of patients. In the two patients in whom there was evidence of active signalling through the BCR, proliferation rates were at the higher end of the range (>0.3 %/day); however, the two patients with the highest proliferation rates did not exhibit active BCR signalling.

Strikingly the patients with the highest proliferation rates (K8 and K10) harboured the cytogenetic abnormality Trisomy 12 and expressed CD38 (12 % and 51 % respectively). It has recently been reported that in addition to increased expression of CD11a, CD49d and CD38 (D'Arena et al., 2001; Su'ut et al., 1998; Zucchetto et al., 2013), Trisomy 12 cells have increased expression of CD11b, CD18, CD29 and ITGB7; they exhibit upregulated integrin signalling and enhanced VLA-4 directed adhesion and motility (Riches et al., 2014). The heterodimeric integrins CD11a/ CD18 (LFA-1), CD11b/CD18 (Mac-1) and CD49d/CD29 (VLA-4) are transmembrane proteins that are involved in the transendothelial migration of leukocytes from the peripheral blood into the lymph-node compartment. In an *in-vitro* circulation model of CLL, it was found that cells that underwent transendothelial migration expressed CD38 and CD49d (Walsby et al., 2014). Furthermore, Pasikowska et al have recently reported that in addition to identifying CLL cells with the greatest potential to undergo transendothelial migration, CD49d expression identifies CLL cells with enhanced capacity to activate CD4 and CD8 T cells (Pasikowska et al., 2016).

Our finding that the patients with the highest proliferation rates expressed CD38 is consistent with that of Calissano et al who found that the fraction of CD38 positive cells is enriched for proliferating cells (Calissano et al., 2009). It is therefore feasible, that enhanced migration into the tumour microenvironment, in addition to enhanced

capacity to activate CD4 T cells, contributed to the increased CLL proliferation rates in these patients.

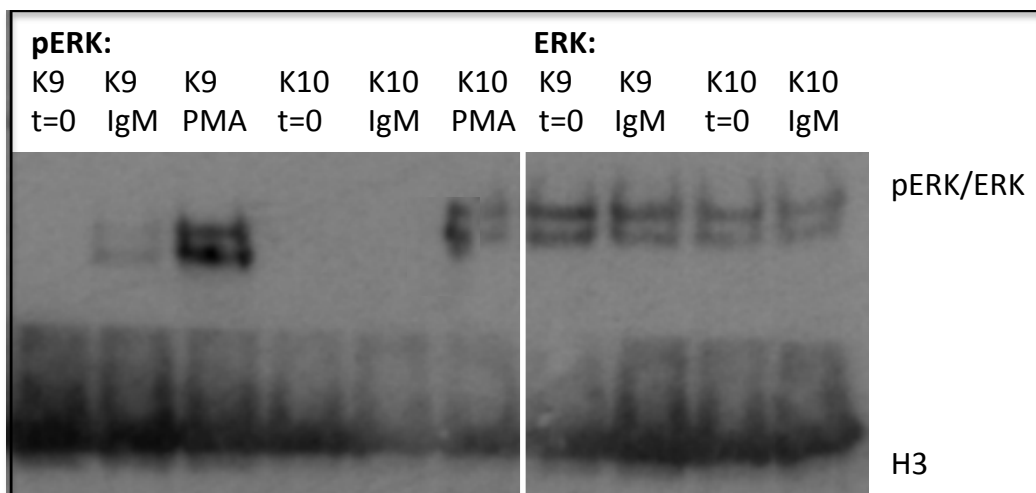


Figure 5.8 Representative Western Blot illustrating pERK activation

Phospho-ERK and total-ERK were measured by Western Blotting at baseline, following at baseline and following 10 minutes stimulation with agonistic anti-IgM in patients K9 and K10. PMA was used as a positive control for activation. Histones (H3) were probed as a loading control.

Table 5.2 Summary of sIgM expression, ERK phosphorylation and CD38 expression and CLL proliferation rate in patients with mutated *IgV_H* genes

Patient	sIgM (MFI)	Stim/ Unstim pERK	Stim/ Unstim ERK	Activation ratio	CD38 (%)	Proliferation rate F(%/day)
K1	924	1.02	0.83	1.22	7%	0.08
K2	1047	2.86	0.99	2.86	0%	0.33
K3	242	0.21	0.71	0.3	1%	0.125
K4	2534	0.16	0.82	0.21	0%	0.215
K5	410	1.63	1.71	0.95	0%	0.190
K6	507	0.56	0.97	0.58	3%	0.230
K7	183	1.14	0.95	1.2	0%	0.280
K8	152	0.59	0.7	0.84	12%	0.95
K9	512	4.73	1.01	4.69	0%	0.32
K10	139	1.14	0.81	1.4	51%	0.72

5.4.4 Correlation of proliferation rate with telomere length

Telomere length has been reported to correlate with clinical stage, *IgV_H* mutational status, CD38 expression, poor risk cytogenetics and β 2-microglobulin levels in CLL (T. T. Lin et al., 2010; Roos et al., 2008). Furthermore, although *in-vivo* deuterated water studies did not demonstrate a statistically significant correlation between proliferation rates and biomarkers of poor-risk disease, they reported correlation with progressive CLL. We therefore explored whether there is a direct correlation between telomere length and *in-vivo* proliferation rate (Figure 5.9). Telomere length was measured using the STELA assay (5.3.13), the assay was performed by Prof Baird, our collaborator at Cardiff University. Mean telomere length in our cohort of patients was equal to that reported by Lin et al in patients with Binet Stage A CLL (T. T. Lin et al., 2010). Notably, K9 had the greatest BCR signalling capacity (pERK activation), a proliferation rate above the median of 0.255 %/day and the shortest telomere length.

However, there was no significant correlation between telomere length and proliferation rate in our cohort of patients. Although telomere length has been reported to be strongly predictive of clinical outcome in CLL, it has since been reported that there is no significant telomere attrition over time in CLL cells (CH Jones, verbal communication) suggesting that proliferation rate and telomere length are not directly related to each other.

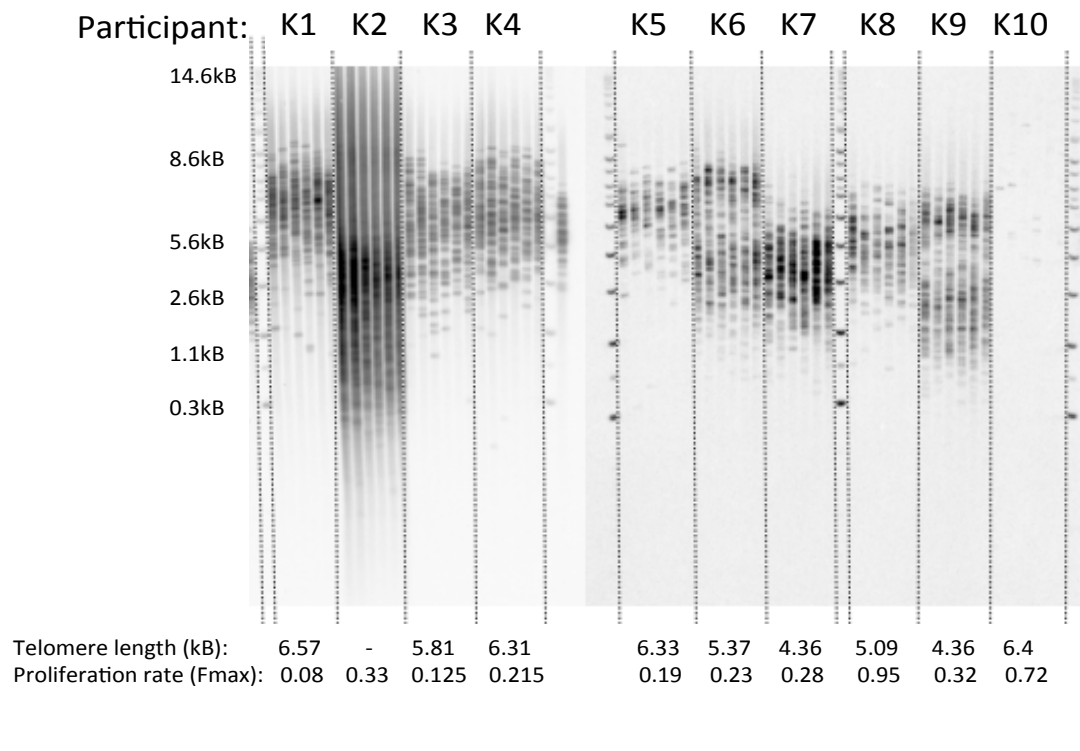


Figure 5.9 CLL Telomere length of patients recruited to the *In-Vivo* Study of Tumour Kinetics in CLL (K1-K10) using STELA

XpYp gels displaying the telomere profiles in CLL cells. Mean telomere length (kb) and proliferation rate (Fmax) are detailed above. STELA was performed by Prof. Baird.

5.4.5 Release rate of deuterium labelled CLL cells into the peripheral blood

The time taken for recently proliferated CLL cells to be released into the peripheral blood from the lymph-node compartment reflects the duration of time that leukaemic cells receive survival and proliferation signalling from the tumour microenvironment. Disruption of this interplay is potentially a desirable approach to inhibit disease progression.

To investigate the time taken for proliferated CLL cells to be released from the lymph-node compartment (LN) into the peripheral blood (PB), phlebotomy was performed daily for the first week and then weekly for four weeks after labelling.

PBMCs were harvested and stored at -180°C for future use. Cryopreserved cells were recovered in RPMI for 60 minutes at 37°C before being surface-stained. Viable CLL cells were flow-cytometrically sorted on co-expression of CD5 and CD19. CLL cells were stored as dry pellets and transferred to St George's Hospital in batches for GC MS analysis of deuterium incorporation. Figure 5.10 illustrates the Cycle 1 labelling profile of CLL cells in all ten participants over the eight-week period. Median time to maximum release was 10.5 days (range = 2-56 days). Time to maximum release of ≥ 14 days was associated with splenomegaly in 4/5 cases. This finding is concordant with observations made using both deuterated water, in which slow release was associated with high CXCR4 expression and progressive disease, and tritiated thymidine studies that showed a distinct profile of labelling in patients with organomegaly (Calissano et al., 2009; Zimmerman et al., 1968). The patient with the longest time to maximum release (K4) had the greatest degree of splenomegaly and the highest sIgM expression (18x higher than the lowest sIgM expression), possibly suggesting that greater tissue retention time leads to greater sIgM expression.

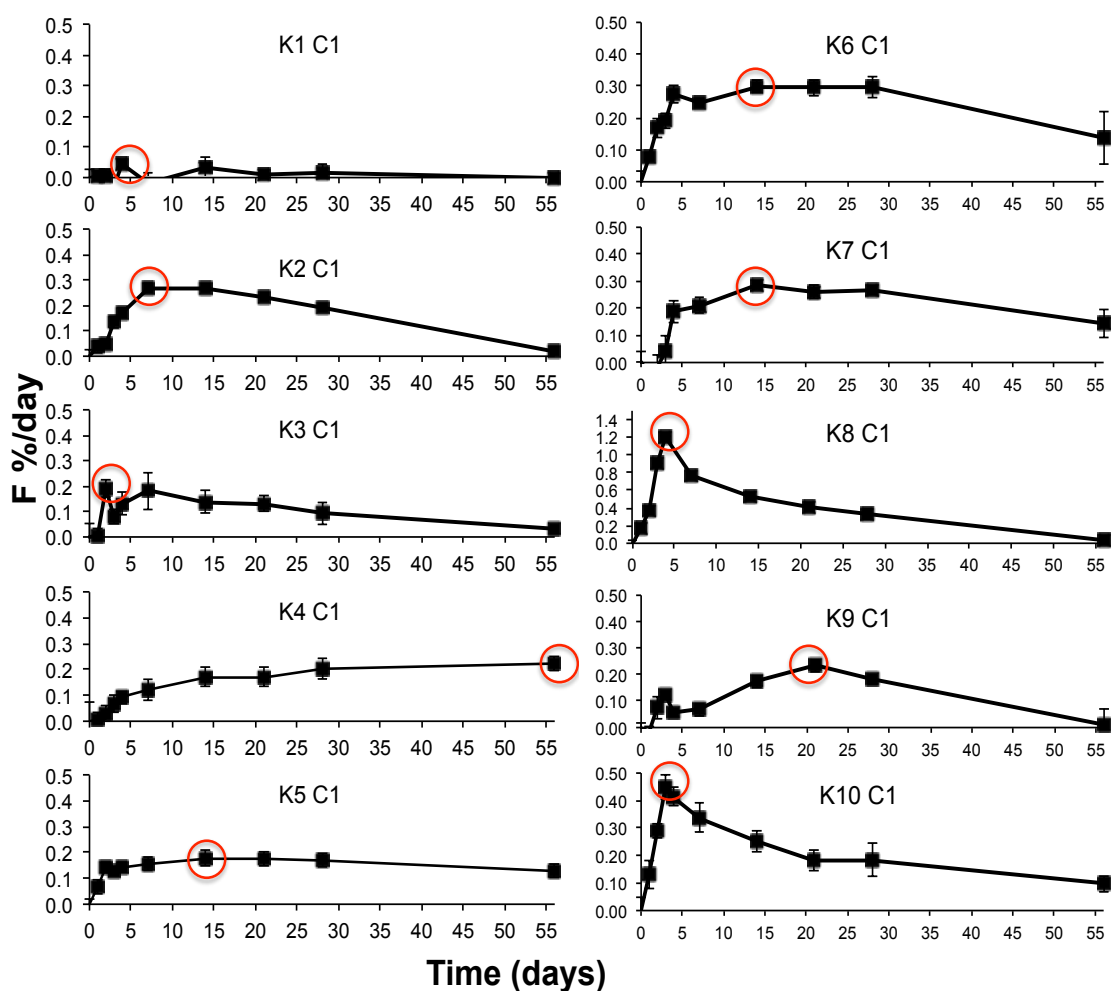


Figure 5.10 Release rates of recently proliferated CLL cells into the peripheral blood.

The fraction of deuterium labelled CLL cells in the peripheral blood during Cycle 1 is illustrated for each participant. Time to maximum release is indicated by a red circle.

5.4.6 Recently proliferated CLL cells can be detected 12 weeks post labelling

An investigation into the rate of disappearance of labelled cells from the peripheral blood, reflecting the loss of cells by apoptosis or migration, was necessary to establish the longevity of recently proliferated CLL cells. Deuterated water studies demonstrated that proliferation is significant in the pathogenesis of CLL but it was not possible to determine disappearance rates due to the ongoing uptake of deuterium into proliferating cells during the 'wash-out' phase. Deuterated glucose studies have the advantage of providing a 'pulse' of clearly defined labelling, allowing the disappearance of labelled cells to be studied.

Rate of disappearance of labelled cells was initially investigated by measuring the fraction of labelled CLL cells in the peripheral circulation weekly for the first four weeks and then after eight weeks. Having observed that the rate of disappearance was very slow, with labelled cells detectable after 8 weeks in half of patients, sampling was extended to twelve weeks. Figure 5.11 illustrates that labelled cells were detected in all 3 patients assessed after 12 weeks. This rate of disappearance is profoundly reduced compared to B lymphocytes, reported to have a half-life of 12 days (Defoiche et al., 2008). Secondly, in patients K8 and K10, there is a rapid loss of labelled cells over the first 4 weeks and then a plateau over the subsequent 8 weeks, suggestive of heterogeneous disappearance rates within the CLL clone. Remarkably, these are the two patients with the highest proliferation rates, CD38 expression and presence of Trisomy 12.

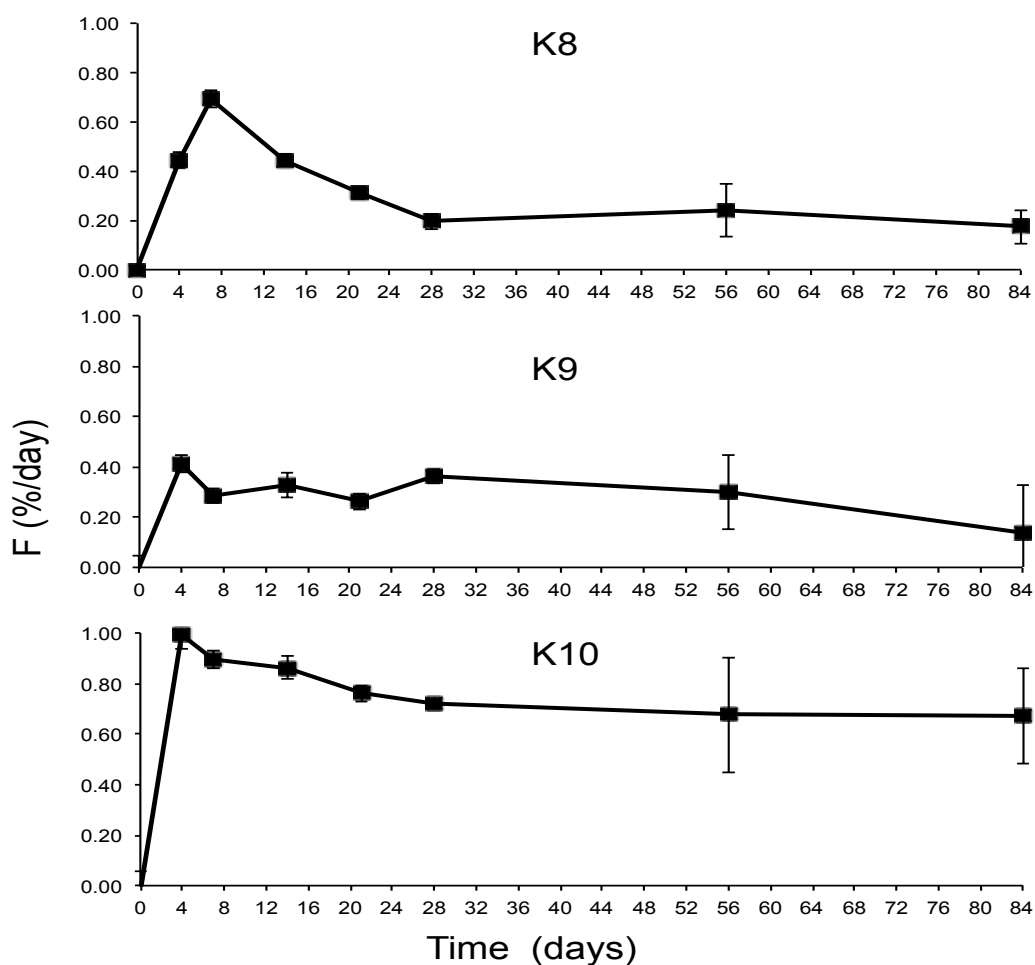


Figure 5.11 Disappearance rates of labelled CLL cells over a 12 week period

Deuterium enrichment in CLL cells was measured on day 4 and day 7 and then weekly for 4 weeks. Late measurements were performed on days 56 and 84 to assess disappearance over an extended time-period in three patients. The fraction of deuterium labelled cells at these pre-specified time-points is illustrated.

5.4.7 Investigation into whether recently proliferated CLL cells re-enter the lymph-node compartment

CLL is considered to be a two compartment disease, where proliferation takes place in the lymph-node compartment, after which cells are released into the circulation where they remain quiescent and a proportion of cells die by apoptosis (Herishanu et al., 2011). Deuterium labelling studies have demonstrated that expression of CXCR4/ CD5 identifies CLL cells at varying stages of proliferation. Cells expressing CD5^{hi}/ CXCR4^{low} represent the most recently proliferated population and it is hypothesised that re-expression of CXCR4 in circulating cells enables them to re-enter the lymph-node via SDF-1 ligation, to under-go a further round of proliferation (Calissano et al., 2009). However, lymph-node re-entry has not been proven.

We sought to investigate the hypothesis that recently-proliferated CLL cells can re-enter the lymph-node by performing serial FNAs in three patients with palpable cervical lymph-nodes. Samples were collected under a previously ethically approved study (REC 09/H0805/5). Lymph-node sampling was initially performed on days 1, 7, and 21 post labelling to capture peak labelling in proliferating cells, maximum release from the lymph-node and possible re-entry back to the lymph-node compartment. Sampling time was informed by peripheral blood CLL labelling (Cycle1) and was adjusted for each participant in the study.

Paired peripheral blood (PB) and lymph-node (LN) labelling for the entire CLL population for the three participants is illustrated in Figure 5.9. Deuterium labelling in LN derived CLL cells was significantly greater than labelling in PB CLL cells drawn on Day 7 in patients K2 and K6, reflecting that CLL cells proliferate in the LN in these patients. Comparison of PB in LN labelling in K6 confirmed the slow release rate, demonstrating that it took 28 days for labelling to equilibrate between the two compartments. In Patient K4, who had significant splenomegaly, there was no deuterium labelling detected on Day 7 but labelled cells were detected on Day 28, suggesting that recently proliferated cells had entered the lymph-node from the peripheral blood.

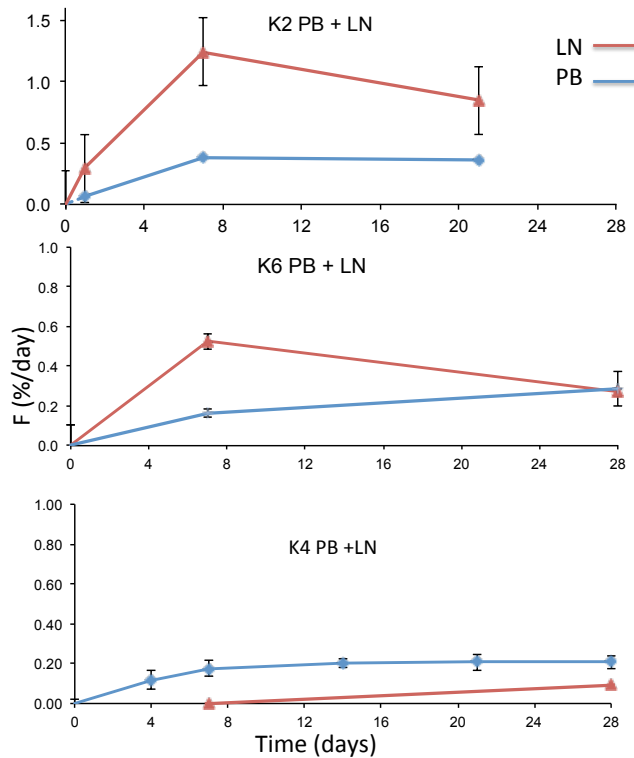


Figure 5.12 Paired lymph-node and peripheral blood deuterium labelling

Paired lymph-node and peripheral blood sampling was performed on three patients. Viable CLL cells were FACS sorted and deuterium enrichment measured by GC MS.

5.4.8 Sub-populations of different proliferative capacity exist in the CLL lymph-node

Deuterated water studies performed by Calissano et al demonstrated that the CXCR4^{lo}/ CD5^{hi} sub-population in peripheral blood was enriched for recently proliferated CLL cells (Calissano et al., 2009). It is thought that these cells undergo a dynamic phenotypic change in the circulation that enables their recirculation to the lymph-node. We therefore investigated whether CLL cells of distinct proliferative capacities exist in the lymph-node. Analysis of sub-populations defined by CXCR4 and CD5 expression in paired samples from peripheral blood and lymph-node was possible in one patient. Deuterium enrichment in the entire CLL population (black) was measured in relation to that in sub-populations that were flow sorted according to CXCR4 and CD5 expression. Figure 5.13a and b demonstrate that although not directly comparable with those seen in the peripheral blood, sub-populations with distinct

proliferative characteristics exist in the lymph-node. The observation that deuterium enrichment is similar in the sub-population defined by CXCR4 low/CD5 high is similar to that of the entire CLL population suggests that this sub-population accounts for the bulk of CLL cells in the lymph-node and that the unlabelled population characterised by CXCR4hi/ CD5low is a minor population. On day 28, labelling was lost from the lymph-node population of cells with CXCR4low/ CD5hi, and was gained in the intermediate population, possibly indicating re-entry of cells with an intermediate phenotype.

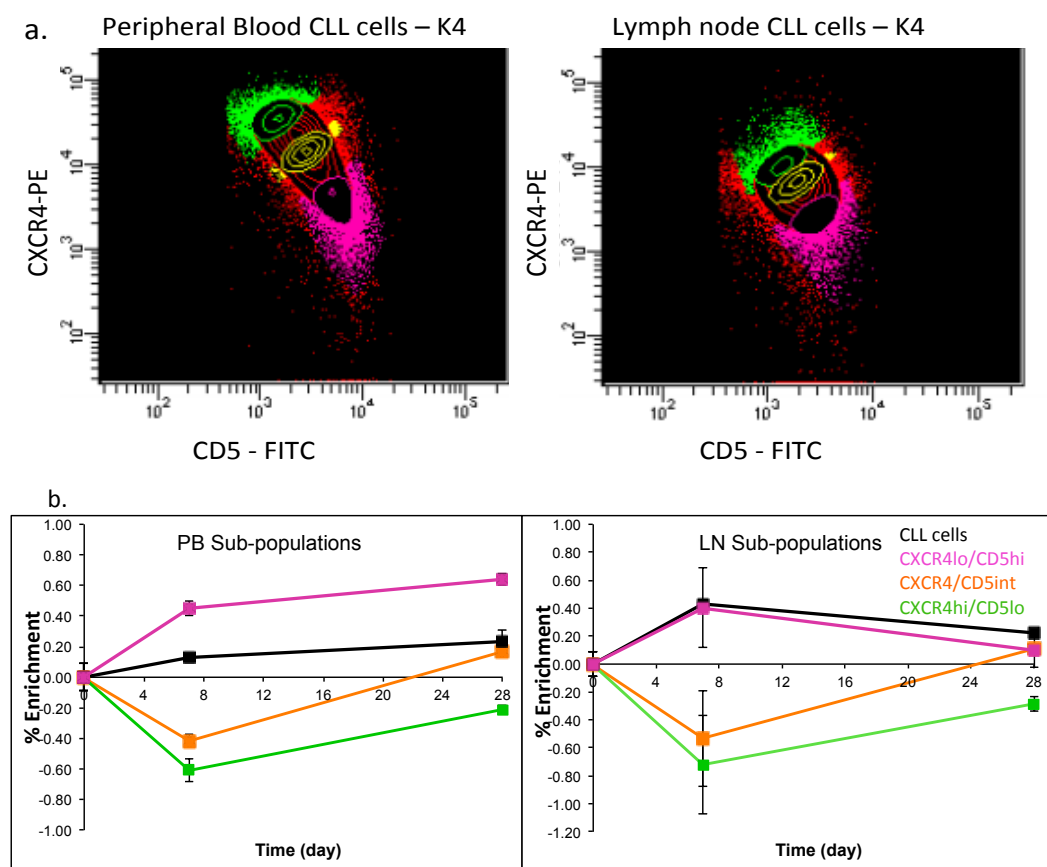


Figure 5.13a FACS scatter plot of lymph-node and peripheral blood derived CLL cells

Viable CLL cells were FACS sorted according to co-expression of CD19 and CD5. Expression of CXCR4 and CD5 were measured to ensure that populations were phenotypically distinct.

Figure 5.13b Deuterium enrichment in peripheral blood and lymph-node CLL populations

Deuterium enrichment in the entire CLL population (black) and sub-populations defined by CXCR4 and CD5 on days 0, 7 and 28 post labelling. Deuterium enrichment was measured using GC-MS.

5.4.9 Subclonal heterogeneity identified by CXCR4/ CD5 expression reveals a quiescent sub-population that does not appear to be derived from a recently proliferated population

Calissano et al have previously reported that expression of CXCR4 and CD5 identifies the most recently proliferated sub-population in CLL, a critical finding that demonstrates a relationship between trafficking and proliferation. They proposed a model whereby CXCR4 is re-expressed and CD5 is reciprocally lost by circulating CLL cells, which subsequently migrate to the lymph-node compartment to undergo a further round of proliferation. Using the 'pulse-chase' labelling, possible only with deuterated glucose, we were able to investigate the kinetics of CXCR4/ CD5 expression and the relationship between sub-populations of CLL cells.

Our aim was to establish whether the peripheral blood compartment in CLL is homogenous, with all cells having an equal potential to undergo proliferation determined by migration into tissue sites or whether sub-populations are functionally distinct.

Deuterium labelled peripheral blood CLL cells from participants K2, K3 and K4 were flow-cytometrically sorted according to expression of CXCR4 and CD5 at ten time-points over eight weeks. Deuterium labelling was analysed by GC MS. In all three patients, the sub-population with CXCR4^{hi}/CD5^{low} remained unlabelled/minimally labelled throughout the eight-week period post labelling. This suggests that this sub-population is quiescent and independent of the other populations.

As previously reported, the sub-population with CXCR4^{low}/ CD5^{hi} demonstrated the greatest degree of labelling (3.5- 6 x that of the CLL population as a whole) (Calissano et al., 2009). Furthermore, as labelling was lost from this population, it was reciprocally gained in the population of CLL cells with intermediate expression of CXCR4/CD5, suggesting that that these populations are in continuity with each other (Figure 5.14).

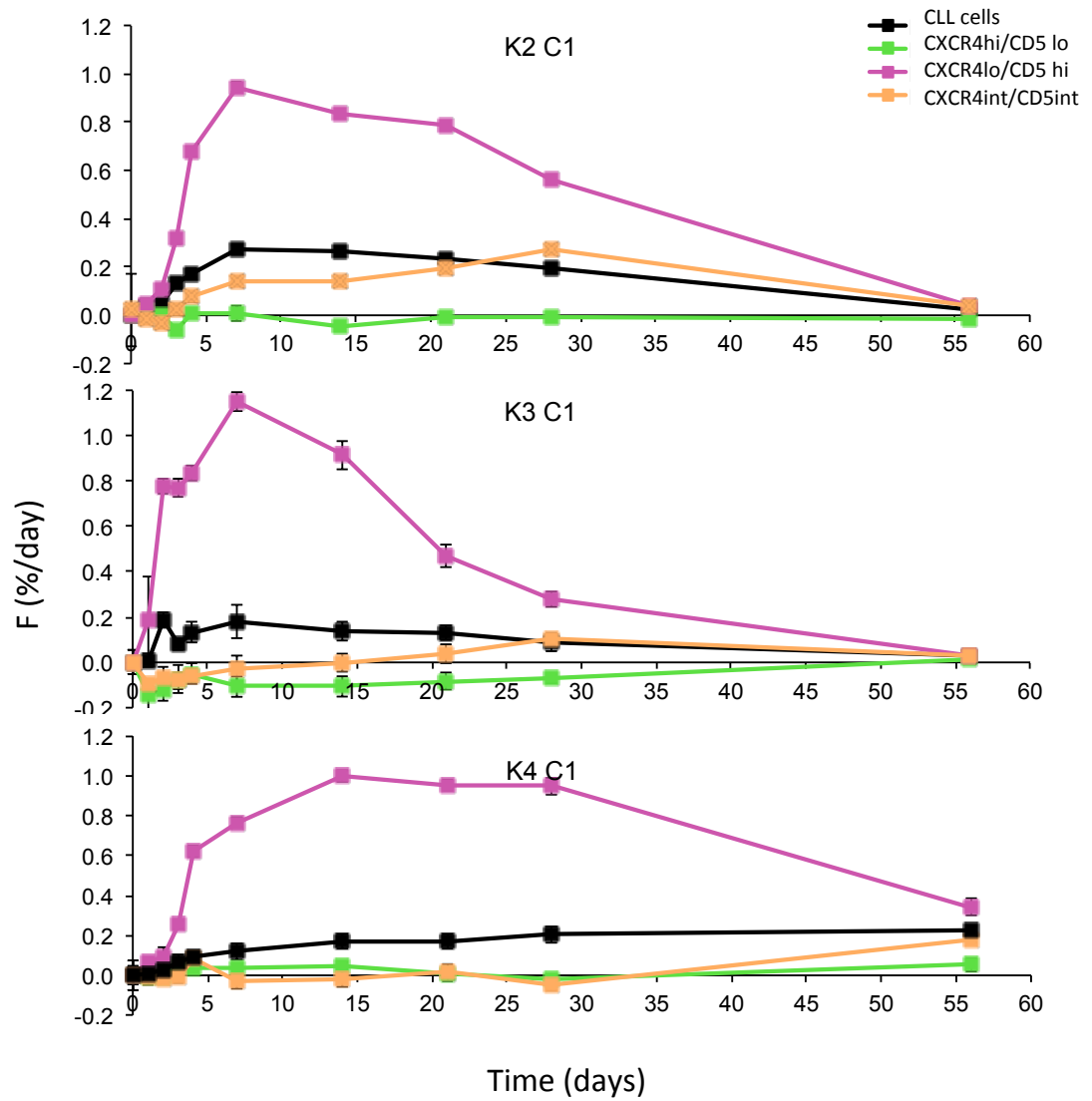


Figure 5.14 Deuterium labelling in CLL sub-populations defined by CXCR4/ CD5 expression over 56 days

Labelled CLL cells were FACS sorted according to CXCR4/ CD5 expression over an eight week period post *in-vivo* labelling and deuterium enrichment measured by GC MS. Sub-populations are shown relative to the unsorted CLL population (black line).

5.4.10 Recently proliferated CLL cells are characterised by high sIgM expression that is subsequently down-regulated in circulating CLL cells

Having confirmed the relationship between trafficking and proliferation in CLL and having identified that there is a quiescent sub-population defined by CXCR4^{hi}/ CD5^{low} expression, we went on to investigate the relationship between sIgM expression, proliferation and trafficking. Expression of sIgM has previously been linked to BCR signalling in patients with mutated *IgV_H* genes (Mockridge et al., 2007) but the relationship with proliferation, fundamental to pathogenesis, has not been investigated.

A previous investigation into functional sub-clonal heterogeneity in CLL used sIgM expression to define sub-populations. The authors reported that the sub-population with the lowest sIgM expression was enriched with markers of recent activation (CD25, CD38) and that there was a trend towards increased Ki67 expression (n=2) suggesting evidence of recent proliferation (Coelho et al., 2013). However, at odds with this was their observation that CD5 expression, a marker of recent BCR signalling, was increased in the sub-population with the highest sIgM expression. Characterisation of the relationship between sIgM expression, proliferation and trafficking is essential in view of the role of BCR signalling in CLL.

We flow-sorted labelled CLL cells from three patients according to sIgM expression, taking the lowest, intermediate and highest 10 % cells at pre-specified intervals over an eight week period post labelling. Figure 5.15 illustrates that CLL cells with the highest sIgM expression have the greatest deuterium labelling, ie have recently proliferated. This is most profound in Patient K8, where the sIgM Hi population has >30 x deuterium labelling of the sIgM Low population. This patient had that highest proliferation rate overall but a low median fluorescent intensity of sIgM expression, further emphasising the subclonal nature of the disease.

Labelling is rapidly lost from the population with the highest sIgM expression relative to the CLL population as a whole, possibly due to rapid dilution of this compartment with unlabeled CLL cells.

Cells with the lowest sIgM expression exhibit minimal deuterium labelling, suggesting that this population is quiescent and not derived from sIgM Hi population i.e similar to the CXCR4hi/ CD5 lo sub-population.

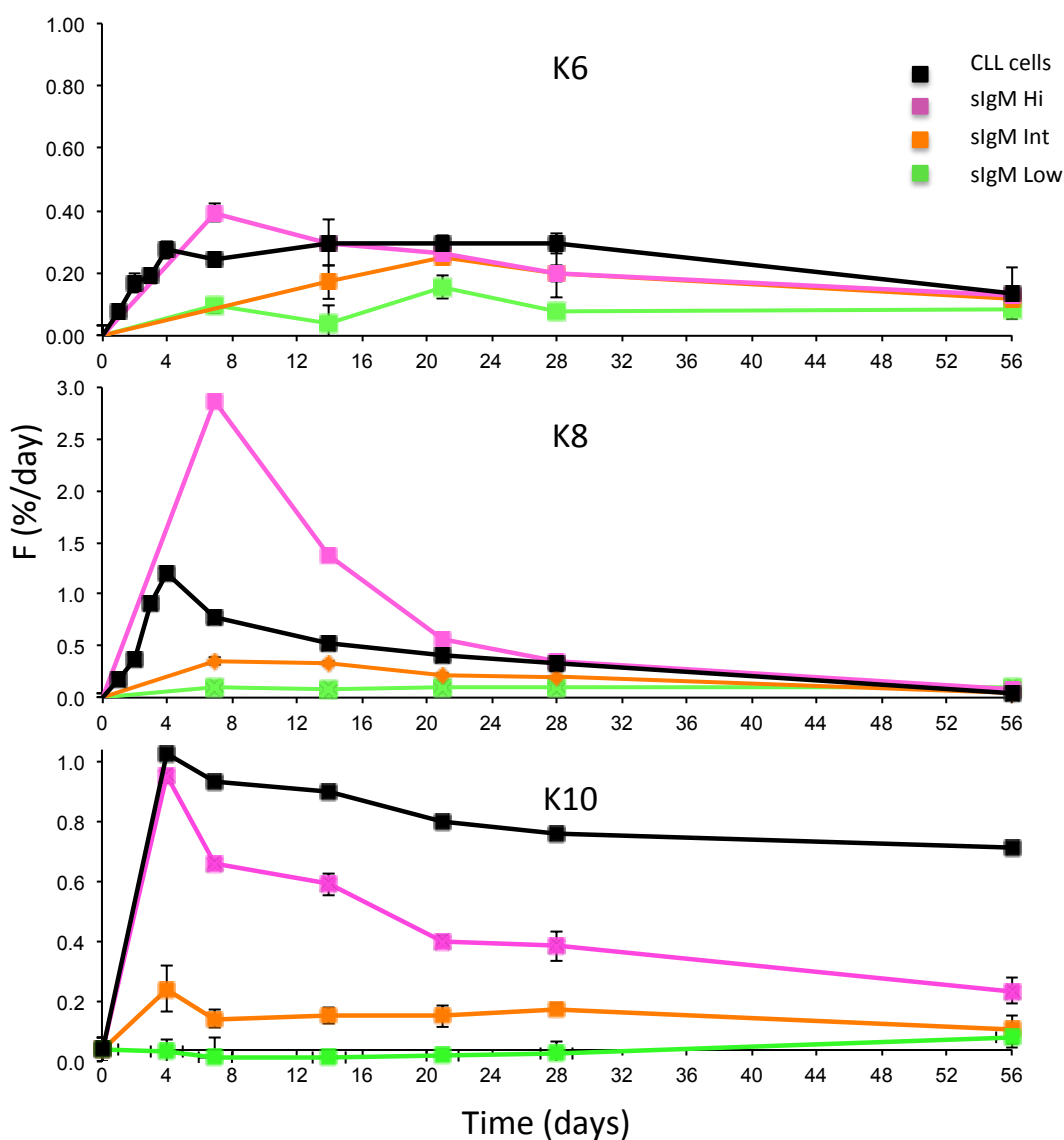


Figure 5.15 Deuterium enrichment in entire CLL population and sub-populations defined according to sIgM expression

CLL cells were labelled *in-vivo* (n=3) and viable CLL cells were sorted according to sIgM expression, taking top (sIgM Hi), middle (sIgM Int) and bottom (sIgM Lo) 10 % cells. Deuterium enrichment was analysed by GC MS.

5.4.11 BCR internalisation identifies CLL sub-populations with distinct proliferative capacities

In view of the observation that sub-population of CLL cells with the highest degree of sIgM expression represented the most recently proliferated population, and that it has been found that sIgM expression is the principal factor that determines BCR internalisation following agonistic sIgM ligation (Coulter et al manuscript submitted), we went on to investigate the relationship between BCR internalisation and proliferation. Deuterium labelled CLL cells were FACS sorted according to BCR internalisation, taking the highest, intermediate and lowest 10 % cells. BCR internalisation was measured using the pHrodo™ assay described in 5.3.11. Figure 5.16 illustrates that the 10 % CLL cells with the highest BCR internalisation is enriched with proliferated cells whilst the 10 % cells with the lowest BCR internalisation remain unlabeled/ minimally labelled. This pattern mirrors the relationship between sIgM expression and proliferation and suggests a pathway whereby sIgM expression, BCR internalisation and proliferation are linked. This is consistent with the finding that lymph-node derived CLL cells and those expressing high levels of CD5 have the greatest capacity to internalise the BCR (Coulter, manuscript submitted).

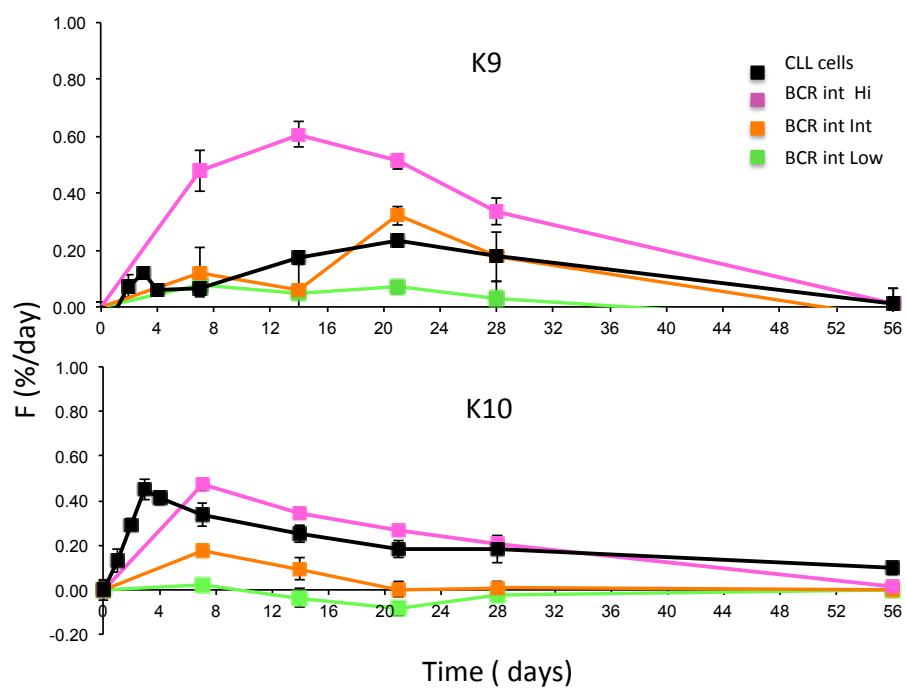


Figure 5.16 Deuterium enrichment in entire CLL population and sub-populations defined according to BCR internalisation

CLL cells were labelled in-vivo (n=2) and viable CLL cells were sorted according to BCR internalisation, taking top (BCR int Hi), middle (BCR int Int) and bottom (BCR int Lo) 10% cells. Deuterium enrichment was analysed by GC MS.

5.4.12 Sub-clonal heterogeneity in telomere length

Having identified a seemingly quiescent subpopulation of CLL cells characterised by CXCR4hi/ CD5lo, low sIgM expression and low BCR internalisation, we investigated telomere length in sub-populations, taking the highest and lowest 10 % according to BCR internalisation. Our hypothesis was that if the functional properties of this cohort of cells were fixed, telomere length would inversely correlate with the proliferation rate. Figure 5.17 illustrates telomere length (measured by STELA) in sub-populations defined by BCR internalisation. In one of three patients (K10), telomere length was shortened in the sub-population with the highest BCR internalisation. Additionally, the clonal proliferation rate in this patient was more than 2 x that of the two patients in which no difference in telomere length was observed.

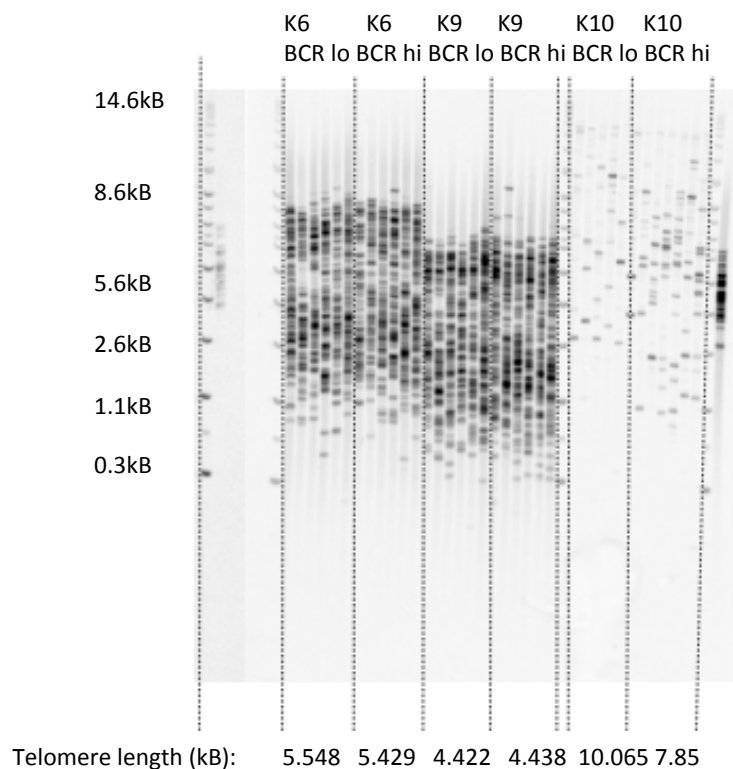


Figure 5.17 Telomere length in CLL cells according to BCR internalisation

CLL cells were flow sorted according to ability to internalise the BCR. The highest and lowest 10 % cells were collected and DNA extracted for telomere length analysis using the STELA assay. XpYp gels display the telomere profiles in CLL cell subpopulations according to BCR internalisation. Mean telomere length (kb) is detailed below.

5.5. Discussion

The purpose of this investigation was to use *in-vivo* labelling to further characterise the relationship between trafficking and proliferation that underpins the clinical heterogeneity in CLL.

The *In-Vivo* Kinetics Study was successful in recruiting to target (n=10) and collecting data at all pre-specified time-points, amounting to 16 hospital attendances per participant. We set out to recruit a cohort of patients with early-stage, non-progressive CLL in whom kinetics studies could be performed over a sixteen-week period. This specification unintentionally resulted in the recruitment of a cohort of patients with M-CLL. Although this enabled the interrogation of a seemingly biologically 'homogeneous' cohort of patients, findings from this study are only applicable to patients with M-CLL. A major amendment has been agreed to enable the recruitment of patients with UM-CLL to an abbreviated period of study. Importantly, within our cohort of patients, three expressed CD38 at $\geq 7\%$; discordance being associated with an intermediate clinical prognosis (Hamblin et al., 2002).

First, we measured the clonal proliferation rate in each patient; the proliferation rate in our study was highly heterogeneous (0.08 %/day - 0.95 %/day) and the mean proliferation rate was lower than that previously reported by Messmer et al, consistent with the clinical profile of our cohort. In contrast, the 'Messmer' cohort of patients included more patients with adverse prognostic features; 50 % had UM-CLL, expressed CD38 and exhibited progressive disease (Messmer et al., 2005).

There was excellent intra-patient correlation in proliferation rates, demonstrating that deuterated glucose labelling could be used to measure the effect of a drug on *in-vivo* proliferation in clinical trials. In one exceptional patient, who had a small clone size and had recently been diagnosed with CD38+ CLL, there was an apparent doubling in proliferation rate between cycles 1 and 2.

An investigation into the relationship between BCR expression, BCR signalling, CD38 expression, proliferation and telomere length was necessary in view of recent

translational studies that have emphasised the importance of the BCR axis in disease pathogenesis. Importantly, we found that the highest proliferation rates were associated with CD38 expression and trisomy 12. Expression of CD38 has previously been associated with increased propensity to migrate into the tumour microenvironment, where cells receive pro-survival signalling and undergo proliferation (Vaisitti et al., 2010). Trisomy 12 is associated with increased expression of CD11a, CD38, CD49d, the latter being strongly associated with trans-endothelial migration and capacity to activate T cells in the lymph-node compartment (D'Arena et al., 2001; Pasikowska et al., 2016; Su'ut et al., 1998; Zucchetto et al., 2013). In the two patients with trisomy 12, sIgM expression was low and there was no evidence of pERK activation in response to BCR ligation, suggesting that BCR signalling was not responsible for the higher proliferation rates in these patients.

There was no direct relationship between sIgM expression, BCR signalling in response to agonistic sIgM ligation and proliferation rate in our cohort of patients with M-CLL and this remains an outstanding area of investigation in patients with UM-CLL. However, not all patients displayed anergy to BCR ligation; two patients exhibited activation of pERK in response to BCR ligation; one of these patients had a high level of sIgM expression (K2) and proliferation rates in both patients were above the median (0.255 %/day). Telomere length, only measurable in one of the two patients, was the shortest in our cohort – possibly suggesting a relationship between BCR responsiveness and sustained proliferation. However, a larger study is warranted to investigate this further.

Our study was the first to formally study release and disappearance rates of labelled CLL cells in a cohort of patients with well-defined biological characteristics. Release rates were heterogeneous and surprisingly slow, particularly in patients with splenomegaly. The patient with the slowest release rate (K4) had the largest spleen and significantly higher sIgM expression. Slow appearance of labelled cells, in the context of splenomegaly, was reported in a very early study of tumour kinetics in CLL that used *in-vivo* tritiated thymidine labelling (Zimmerman et al., 1968) and more recently in deuterated water studies, where late appearance was associated with high

CXCR4 expression. One explanation for slow release in patients with splenomegaly is increased tissue retention resulting from interaction between CXCR4 on CLL cells and SDF-1 expressed by stromal cells. Expression of S1P1, required for leucocyte egress, and reported to be defective in CLL (Capitani et al., 2012; Sic et al., 2014), should be investigated in relation to release rates.

Disappearance rates were heterogeneous both between and within patients suggesting the existence of sub-populations with variable rates of cell turn-over. More specifically, the initial rapid decline may arise from a population of cells with a fast rate of cell-turnover whilst the subsequent slower rate of decline may result from a population with a slow rate of proliferation and cell-death. The observation that labelled cells were detected after 12 weeks in the three patients who underwent an extended period of monitoring suggests that significantly reduced rates of apoptosis compared to healthy B lymphocytes may contribute to pathogenesis in patients with M-CLL.

Calissano et al have previously demonstrated that recently proliferated CLL cells are characterised by low CXCR4 and high CD5 expression (Calissano & Damle, 2011). We confirmed this finding and by sorting cells according to CXCR4/ CD5 expression at pre-specified time-points over an eight-week period, we found that there was continuity between CXCR4^{lo}/ CD5^{hi} and intermediate populations (CXCR4^{int}/ CD5^{int}), consistent with CXCR4 re-expression in the circulation. However, most strikingly, the decade of cells with the highest CXCR4/ lowest CD5 remained unlabeled throughout the eight-week period of investigation. This suggests that recently proliferated cells re-express CXCR4 to an intermediate level but not to a high level, to re-enter the lymph-node. This goes against the hypothesis that recently proliferated CLL cells maximally re-express CXCR4 to enable lymph-node re-entry and suggests that this population is an independent sub-population, fixed in a state of quiescence.

To investigate the proposed model of lymph-node re-entry, we performed paired peripheral blood and lymph-node sampling over the three weeks post labelling. In one patient, there was increased labelling in the lymph-node compartment between days 7 and 28, consistent with migration of recently proliferated leukaemic cells. However,

this patient was unusual in that peripheral blood labelling was greater the lymph-node labelling and that the prominent clinical feature was of splenomegaly. Studies of proliferation in the spleen are few but it has been reported that proliferation centres do exist in spleens of patients with CLL (Lampert, Wotherspoon, Van Noorden, & Hasserjian, 1999), and it is possible that the spleen was the major site of proliferation in this case. Again, this study should be extended to a larger cohort of patients to further investigate trafficking between the peripheral blood and lymph-node compartments.

To further interrogate the relationship between CLL sub-populations defined by CXCR4 and CD5 expression and trafficking between the peripheral blood and lymph-node compartments, we went on to measure deuterium enrichment in lymph-node derived sub-populations. Importantly, we found that lymph-node derived CLL sub-populations had diverse characteristics of proliferation. The decade of cells with the highest CXCR4 and lowest CD5 expression remained unlabelled over a 28-day period ie appeared quiescent. However, the decade of cells with the reciprocal phenotype had the greatest degree of labelling and as this was comparable with the labelling of unsorted CLL cells, suggests that a large proportion of cells in the lymph-node are CXCR4^{low}/CD5^{hi} and are proliferating. It is notable that on day 28, there was a loss of labelling in the CXCR4^{low}/CD5^{hi} population and a gain in labelling in the intermediate population, possible reflecting lymph-node re-entry of cells that have re-expressed CXCR4 to an intermediate level in the circulation. This is consistent with the observed continuity between these populations in the peripheral blood.

Our investigation into the dynamics of sIgM expression in recently proliferated cells yielded two important findings. First and foremost, we found that recently proliferated CLL cells are consistently characterised by high sIgM expression and that labelling is rapidly lost from this compartment. This is in agreement with the observation that sIgM expression in the lymph-node is greater than in the peripheral blood (Coulter, manuscript submitted) and with recent reports that sIgM is upregulated in response to IL4 signalling in the CLL lymph-node (Aguilar-Hernandez et al., 2016; Guo et al., 2016). However, it challenges the model proposed by Coelho et al that the BCR is internalised

in the lymph-node and that recently proliferated cells are characterised by low sIgM expression and unresponsiveness to BCR ligation (Coelho et al., 2013). Secondly, the sub-population with the lowest sIgM expression appeared as an independent sub-population with minimal deuterium labelling throughout the period of investigation, similar to the quiescent sub-population characterised by CXCR4hi/ CD5 low expression.

Finally, we went on to investigate the relationship between BCR internalisation (reflective of BCR function) and proliferation and the dynamics of BCR internalisation over time. Consistent with the enrichment of the sIgM high fraction with proliferating cells, the cohort of cells with the greatest BCR internalisation exhibited the greatest degree of proliferation. Labelling was rapidly lost from the decade of CLL cells with the greatest BCR internalisation, suggesting that there is rapid dilution of this population with unlabelled proliferating cells. Again we showed that there was an independent sub-population of cells characterised by low BCR internalisation.

By tracking the labelling in sub-populations of cells defined by CXCR4/ CD5 hi, sIgM and BCR internalisation, we were able to investigate the phenotypic changes that recently proliferated cells undergo over an eight-week period. Critically, we identified a discrete, quiescent sub-population characterised by CXCR4hi/ CD5low, low sIgM expression and low BCR internalisation in our cohort of patients with M-CLL. Our limited telomere length analysis found that in one patient (with the second highest proliferation rate), telomere length was shortest in the sub-population with the highest BCR internalisation, suggesting that this cohort of cells has undergone significantly more proliferation. The studies into the relationship between phenotype, function, proliferation and telomere length were limited to a small number of patients and a larger cohort study is necessary to further investigate our preliminary findings.

The size of the quiescent population was not determined in this investigation in which deuterium labelling was measured in fixed populations each accounting for 10 % of the total population. However, when looking at deuterium enrichment in the three sub-populations relative to the entire CLL population, the degree of dilution of the highly labelled population suggests that the quiescent phenotype accounts for a large proportion of cells. The exact size of proliferating and quiescent populations is likely to

be heterogeneous and could be more accurately estimated by measuring deuterium enrichment in all ten decades of cells within the CLL clone.

The identification of a population of quiescent CLL cells is relevant to the observation that patients with M-CLL treated with the BCR antagonist ibrutinib, frequently develop a persistent lymphocytosis and in whom the persisting lymphocytes are characterised by constitutive ERK phosphorylation, and Ki67 negativity (Woyach, Smucker, et al., 2014c). Furthermore, the observation that mean CLL cell expression of CXCR4 increases as patients respond to ibrutinib (Herman, Niemann, et al., 2014b) could be interpreted as enrichment of the sub-population of CXCR4^{hi}/ CD5^{low} cells as the population with the opposing phenotype is eliminated. *In-vivo* labelling studies using deuterated glucose to study the kinetics of proliferating cells in patients commencing treatment with ibrutinib are warranted.

Full phenotypic, functional and genetic characterisation of the quiescent sub-population is now necessary to establish the biological properties of these cells. This investigation should include assessment of features of anergy i.e. constitutive activation of NFAT and pERK and ability to 'signal' in response to agonistic sIgM ligation. Ultimately, we need to establish whether this population is genetically distinct; this requires assessment of the mutational profile using next generation sequencing (NGS).

Finally, we propose a model of CLL trafficking whereby recently proliferated CLL cells released from the lymph-node are characterised by CXCR4^{low}/ CD5^{hi}, high sIgM expression and high BCR internalisation and that within the peripheral blood compartment CXCR4 is re-expressed to an intermediate level, with reciprocal loss of CD5 and sIgM expression. CLL cells that re-enter the lymph-node express an intermediate level of CXCR4 and are derived from the CXCR4^{low}/CD5^{hi} population whilst the CXCR4^{hi}/CD5^{low}/ sIgM^{low} sub-population remains independent in patients with M-CLL.

An investigation into tumour kinetics in patients with UM-CLL is now necessary to determine whether the quiescent sub-population is unique to patients with M-CLL or

whether it is relevant to all patients regardless of *IgV_H* mutational status. The observation that patients with UM-CLL treated with ibrutinib tend not to develop a persistent lymphocytosis of non-proliferating cells, suggests that it may be specific to M-CLL. Secondly, it would be interesting to compare sIgM expression, BCR internalisation, proliferation and disappearance rates in patients with UM-CLL with patients with M-CLL, in whom BCR signalling and proliferation are considered to be less active.

Overall, our detailed investigation using *in-vivo* labelling to study tumour kinetics in CLL provides an important insight into the relationship between phenotypic and functional characteristics of CLL cells and their propensity to migrate and proliferate. This study has contributed to the characterisation of the processes that underpin the clinical heterogeneity in CLL. Further studies are planned to investigate the *in-vivo* mechanism of action of BCR antagonists in CLL.

Chapter 6 . General Discussion

6.1 CLL Pathophysiology

Chronic Lymphocytic Leukaemia is a B cell lymphoproliferative disease characterised by significant biological and clinical heterogeneity where interactions between malignant cells and the microenvironment contribute to disease pathogenesis. Although for many years it was considered to be a disease of failed apoptosis, there is now a wealth of evidence to support the role of proliferation in the biology of CLL. The *in-vivo* labelling study performed by Messmer et al was a pivotal study that demonstrated that in patients with progressive CLL, 'birth-rates' could be as high as 2 % of the malignant clone each day (Messmer et al., 2005). Consistent with this finding was the observation that telomere length is critically shortened in CLL, reflective of a significant replicative history (Lin et al., 2010). More recently, next generation sequencing of leukaemic B cells at sequential time-points has neatly demonstrated that proliferating cells can undergo clonal evolution, particularly in the face of the 'selection pressure' of chemotherapy (Landau et al., 2013; A. Schuh et al., 2012).

6.2 The Tumour Microenvironment

A major contribution to the field of CLL biology was the finding that the proliferation of CLL cells takes place within the lymph-node compartment (Herishanu et al., 2011; Jaksic et al., 2010). Characterisation of the processes that take in the lymph-node compartment has been an area of intense investigation and continues to shed light on the processes that drive the survival and proliferation of CLL cells. Interactions between malignant cells and components of the tumour microenvironment including endothelial cells, stromal cells and nurse-like cells are complex and bidirectional and involve both direct contact and cytokine signalling (Buggins et al., 2008; J. A. Burger et al., 2000; Ghia et al., 2002).

6.3 BCR Signalling in CLL

B cell receptor signalling is most active in the lymph-node and has been shown to be enhanced by IL4 that upregulates CD79b and sIgM expression (Guo et al., 2016). The degree of sIgM expression and BCR signalling in CLL cells is heterogeneous and is closely related to the *IgV_H* mutational status of the patient. In patients with unmutated

IgV_H genes, there is greater sIgM expression and enhanced calcium mobilisation following BCR ligation (Muzio et al., 2008). In patients with mutated *IgV_H* genes, there is a tendency towards 'anergy', where leukaemic B cells are characterised by constitutive activation of NFAT and ERK and failure to mobilise calcium following BCR ligation (Muzio et al., 2008). Clinically, patients with unmutated *IgV_H* genes have a more proliferative disease with a shorter time to first treatment and a shortened overall survival (Damle et al., 1999; Hamblin et al., 1999).

Recently, it has been demonstrated that drugs that target BCR signalling have potent effects both *in-vitro* and *in-vivo* and that in addition to inhibiting signalling downstream of the BCR and reducing proliferation, they impact on the trafficking of CLL cells between the lymph-node and peripheral blood compartments (Herman, Mustafa, et al., 2014a; Herman, Niemann, et al., 2014b). However, the relationship between BCR signalling, proliferation and trafficking of leukaemic cells has not been fully determined. Of note, a small number of patients have failed ibrutinib therapy as a result of expansion of sub-clones harbouring mutations that render cells resistant to the drug (Liu et al., 2015; Woyach et al., 2014b). Full characterisation of the mechanisms of resistance is necessary to develop effective treatments in this cohort of patients.

6.4 The Role of Activated CD4 T Cells in CLL

Our group and others have focused on the role of activated CD4 T cells that contribute to the proliferation of CLL cells in the lymph-node compartment. Patten demonstrated that recently proliferated CLL cells frequently contact activated CD4 T cells and that co-culture of CLL cells with activated autologous T cells leads to the upregulation of CD38 expression and to the proliferation of CLL cells (Patten et al. 2008b; Willimott et al., 2007). Bagnara et al subsequently reported that activated autologous CD4 T cells are required for the engraftment and proliferation of CLL cells in the NSG mouse model of CLL (Bagnara et al., 2011).

The mechanism whereby CD4 T cells activate CLL cells has been shown to be multifactorial. Activated CD4 T cells express CD40L that induces NFκB translocation and leads to enhanced survival, activation and proliferation of CLL cells (Furman et al.,

2000; Hamilton et al., 2012). In addition to direct contact, cytokines including IL4, IL21 and IFN γ have been shown to activate CLL cells and to induce proliferation (Ahearne et al., 2013; Bürgler et al., 2015; Pascutti et al., 2013).

6.5 Manipulation of the Tumour Microenvironment

In view of the fact that T cells appear to play a considerable role in the pathogenesis of CLL, we investigated whether manipulation of the tumour microenvironment by suppressing T cell activity could reduce the proliferation rate of CLL cells. Targeting the microenvironment as opposed to the malignant cells is a novel strategy that is particularly desirable because it should not contribute to clonal evolution and therapeutic resistance.

Ciclosporin (CsA) was selected as the drug of choice due to its potent anti-T cell properties and because of the extensive clinical experience gained in the use of this drug including that in the context of autoimmune complications of CLL. Furthermore, CsA has been reported to have anti-tumour activity in a proportion of patients with CLL (Cortes et al., 2001).

Ciclosporin is a calcineurin inhibitor that inhibits T cell activation by inhibiting the nuclear translocation of NFAT and NF κ B. However, NFAT and NF κ B play a significant role in signalling downstream of the BCR in CLL cells that contributes to disease pathogenesis (Hewamana et al., 2008; Le Roy et al., 2012). Indeed, Apollonio et al demonstrated that inhibition of NFAT-C1 signalling using the cell permeable VIVIT peptide in MEC-1 transplanted xenograft mice reduced the rate of tumour growth and increased survival (Apollonio et al., 2013). We therefore designed a comprehensive series of *in-vitro* assays to determine whether CsA inhibits T cell activation in CLL or whether its effects are due to disruption of BCR signalling. Although it is possible that CsA inhibits signalling from other components of the tumour microenvironment including stromal cells, endothelial cells and nurse-like cells, we considered the T cell: CLL cell axis to be critical and this remained the focus of our investigation.

6.6 In-vitro Studies into the Effect of CsA on CLL B and T cells

CLL T cells have a distinct phenotype, characterised by an inversion of the normal CD4:CD8 ratio, a preponderance of effector memory cells and relative absence of naïve precursors (Görgün et al., 2005; Nunes et al., 2012). We therefore started by investigating the effect of CsA on CLL T cells. Crucially, we observed that CsA inhibits the co-expression of IFN γ and CD69 by CLL CD4 and CD8 T cells. IFN γ has been shown to be a major mechanism whereby CLL-specific Th1 cells upregulate CD38 expression by CLL cells (Bürgler et al., 2015). Secondly, CD69 is a leukocyte activation antigen that plays a role in the retention of CLL cells within the lymph-node compartment (Shiow et al., 2006; Sic et al., 2014). Furthermore, we demonstrated that CsA has a profound effect on the expression of CD40L by CD4 T cells; CD40L has been shown to play a key role in the survival and proliferation of CLL cells (Ghia et al., 2002; Hamilton et al., 2012).

We investigated the effect of CsA on the expression of IL21, a cytokine that has been shown to contribute to the proliferation of CLL cells in response to CD40L activation (Ahearne et al., 2013; Pascutti et al., 2013). IL21 is under the transcriptional control of NFAT and has previously been reported to be sensitive to CsA (Kim et al., 2005). However, we did not demonstrate that IL21 expression is sensitive to CsA and this should be further investigated.

Investigation of the effect of CsA on transcription factor signalling in CLL T cells using image flow-cytometry revealed that both NFAT-C2 and NF κ B signalling is inhibited in the presence of CsA. NFAT-C2 and NF κ B are key regulators of the transcription of IL2, IL4, IL6, IL21, TNF α and IFN γ and CD40L, inhibition of which would be predicted to have a significant detrimental effect on CLL cells in the tumour microenvironment.

Having demonstrated that CsA is effective in inhibiting components of T cell signalling that are considered to play an important role in the biology of CLL, we went on to investigate whether CsA has any direct effect on the activation of CLL cells. Importantly, NFAT-C2 and NF κ B-p65 are upregulated in CLL cells compared to healthy B lymphocytes and inhibition has been shown to reduce the viability of the tumour cells (Apollonio et al., 2013; Hewamana et al., 2008; Le Roy et al., 2012).

We found that the mechanism of activation of CLL cells played a role in determining the sensitivity to CsA. CD40L activated CLL cells were resistant to CsA; nuclear translocation of NFAT-C2 and NFκB-p65 was induced by CD40L and was unaffected by CsA, as was expression of the activation markers CD25 and CD69. However, in CLL cells activated through the BCR, there was a small inhibitory effect of CsA on CD69 expression suggesting that BCR signalling is sensitive to CsA. These findings are consistent with the observation that in healthy B-lymphocytes, proliferation induced by agonistic sIgM is sensitive to CsA whereas proliferation induced by CD40L is resistant (Wortis et al., 1995).

In view of previous reports that inhibition of NFAT signalling using the cell-permeable VIVIT peptide reduces the metabolic activity of CLL cells that are 'signalling-competent' and reverses anergy and induces apoptosis in those that are not signalling-competent, we investigated the effect of CsA on the viability of CLL cells (Apollonio et al., 2013; Le Roy et al., 2012). CLL cells were cultured either alone or as PBMCs to assess whether any effect of CsA on the viability of tumour cells was direct or dependent upon the presence of T cells. Consistent with the literature, we found that the viability of CLL cells was greater when cultured as PBMCs. In the presence of CsA, there was a trend towards reduced viability regardless of the cell-purity, suggesting that any reduction in viability was not dependent on the presence of T cells.

Further investigation into the effect of CsA on CLL viability should take BCR signalling-capacity into account. It is possible that the effect of CsA on the viability of tumour cells is partly determined by whether the cells display constitutive activation of NFAT, which is associated with non-responsiveness to BCR signalling.

In order to determine the effect of CsA on the interaction between CLL B and T cells as may be expected in the lymph-node compartment, we employed a co-culture system whereby CLL cells were co-cultured with activated autologous T cells. Importantly, we observed that CsA lead to a profound T cell dependent inhibitory effect on CLL cell activation. However, in addition to the T cell-dependent effect, there was a smaller but significant direct effect on the expression of CD69 by CLL cells.

In conclusion, the *in-vitro* studies demonstrated that CsA reduces the activation of CLL derived CD4 T cells by inhibition of both NFAT-C2 and NFkB-p65 and that this leads to a secondary reduction in CLL cell activation. This confirmed that manipulation of the tumour microenvironment, in order to disrupt the pathogenesis of CLL, was a valid strategy to test *in-vivo*.

6.7 In-vivo Studies into the Effect of CsA in Patients with Early, Unfavourable Risk CLL

We launched a Phase II study to intensively investigate the effect of CsA on CLL in a small cohort of patients with early-phase, adverse risk disease defined by the expression of CD38 who did not meet the standard criteria for treatment (Hallek et al., 2008). Although this cohort of patients is recognised as having an inferior prognosis, no early therapeutic intervention has been shown to alter the clinical outcome and these patients are managed with a ‘watch and wait’ strategy.

The primary outcome of the study was change in *in-vivo* CLL cell proliferation rate measured using deuterated glucose labelling, the first time that this method has been used to measure drug-effect.

In preparation for the clinical trial, intra-patient variation in *in-vivo* proliferation rates was measured in a purely observational study (*In-vivo* Kinetics in Chronic Lymphocytic Leukaemia). It was considered to be excessive to assess this within the CyCLLe study. Although the initial trial protocol for the observational study included only patients with CD38+ non-progressive CLL, difficulties with recruitment meant that it became necessary to relax the eligibility criteria to include all patients with non-progressive disease. Overall, there was significant correlation of *in-vivo* proliferation rates between the two ‘labelling cycles’ (n=10; r =0.633, p =0.048), providing an excellent foundation to investigate the effect of a therapeutic intervention.

The CyCLLe study was opened at KCH an UHW with the help of the Bloodwise Trials Acceleration Program. However, recruitment to this study was a major challenge. Barriers to recruitment included the limited number of patients with non-progressive, CD38+ CLL and the considerable patient commitment required (number of visits and duration of trial). A trial amendment was made to abbreviate the protocol (Cycle 1

became an optional cycle) and an extra trial site was opened (Southampton General Hospital). Four patients completed the study (one was withdrawn following the development of atrial fibrillation and a myocardial infarction that were considered to be unrelated to CsA therapy).

Baseline proliferation rates amongst the five patients recruited to CyCLLe were lower than the mean proliferation rates reported by Messmer et al. The deuterated water studies, in contrast to CyCLLe, included patients with progressive disease associated with higher proliferation rates (Messmer et al., 2005).

There was no demonstrable effect of CsA on proliferation rates after four weeks of therapy despite CsA levels being maintained within the therapeutic range. CLL is a highly heterogeneous disease so the absence of drug-effect in four patients with diverse biological characteristics is insufficient evidence to conclude that CsA does not reduce proliferation in CLL. Baseline proliferation rates in our cohort of patients with CD38+ CLL were low, making it challenging to demonstrate a reduction in proliferation. Inclusion of patients with CD38+ CLL who additionally had UM-*IgV_H* genes may have selected a more 'proliferative' population. However, such patients frequently have progressive disease that warrants conventional therapy. Also, narrowing the eligibility criteria would have led to further difficulties with recruitment. Secondly, it is feasible that dilution of a sub-population of proliferating cells by a large pool of quiescent cells could mask a change in proliferation. One way of focusing on the proliferating sub-fraction would be to measure deuterium labelling in lymph-node derived CLL cells, although this may be altered by changes in the trafficking of CLL cells in the presence of CsA.

In view of the *in-vitro* findings relating to the effect of CsA in CLL, an alternative explanation for the unchanged tumour proliferation rates includes the possibility that *in-vivo*, CLL cells are activated by CsA insensitive mechanisms involving other components of the tumour microenvironment.

Secondary outcome measures included the toxicity of therapy and clinical responses. Treatment was generally well tolerated; drug-related adverse events were mostly

grade 2 or less and importantly, there were no severe infections. None of the patients studied had an objective clinical response to CsA, consistent with the unchanged proliferation rates. This is in contrast to the report from Cortes et al that approximately 20 % had anti-tumour responses; one third of those being sustained (Cortes et al., 2001). However a short-lived lymphocytosis was observed in 4/5 patients immediately following the commencement of CsA. A possible explanation for this phenomenon is that CsA inhibits CD69 expression in CLL cells activated by either autologous CD4 T cells or through the BCR, and may therefore disrupt the retention of tumour cells in the CLL lymph-node. This is in-common with the observation that patients treated with ibrutinib, there is a rapid inhibition of CD69 expression associated with the release of proliferating cell from the lymph-node into the peripheral blood (Herman, Niemann, et al., 2014b). However, it is hypothesised that this 'egress' is related to down-regulation of CXCR4 expression in the presence of ibrutinib. Expression of CXCR4 by leukaemic B cells could have been measured in patients treated with CsA to further investigate the biological process underlying the lymphocytosis.

Translational studies were performed on peripheral blood derived CLL and T cells to determine the effect of calcineurin inhibition *in-vivo*. We found that there was no statistically significant change in the resting phenotype of CLL or CD4 T cells following *in-vivo* CsA therapy. However, there was a trend towards inhibition of NFAT-C2 and NFkB-p65 translocation and expression of CD25 and CD69 in activated CD4 T cells in the presence of CsA. During *in-vivo* CsA therapy, there was a trend towards reduced activation of CLL cells when co-cultured with activated autologous CD4 T cells, suggesting that CsA may inhibit the ability of CD4 T cells to activate CLL cells *in-vivo*. Additionally, there was a trend towards reduced NFAT-C2 and NFkB-p65 nuclear translocation and CD69 expression in CLL cells activated with CD40L, suggestive of reduced responsiveness to activation.

In conclusion, the *in-vitro* studies provided evidence for a two-pronged effect of CsA in CLL; with both T cell dependent and direct effects on CLL cell activation that were inhibited in the presence of CsA. The *in-vitro* studies, in addition to previous reports of the anti-tumour effect of CsA *in-vivo* lead to the justification of a small cohort study to intensively investigate the effect of CsA in early-stage adverse risk disease (CyCLLe). Manipulation of the tumour microenvironment to inhibit proliferation of malignant cells was a novel approach, justified by the wealth of evidence for the role of activated autologous T cells in the pathogenesis of CLL.

Recruitment was the greatest challenge to the success of this study and the small number of patients who completed studies limited the significance of the findings. Baseline *in-vivo* proliferation rates were low and remained unchanged after four weeks of CsA therapy suggesting that this approach of manipulating the tumour microenvironment may not be effective in patients with early stage, adverse risk disease.

It is notable the German CLL Study Group (DCLLSG) has also recognised that the standard approach of 'watchful waiting' in patients with early-phase, adverse risk disease should be challenged. In view of the clinical heterogeneity in this population, the GCLLSG has recently launched a multi-centre, phase three, placebo controlled trial (The CLL 12 trial) where patients with intermediate to very high risk Binet stage A CLL are randomised to ibrutinib or placebo. The risk is calculated according to the comprehensive CLL score that takes into account: age, stage, performance status, serum β_2 M, serum thymidine kinase, mutational state of *IgV_H* genes and presence of adverse risk FISH. Interestingly, CD38 expression (>30 %) was not found to be predictive of overall survival in univariate analysis and was therefore excluded from the prognostic score. The results of this study will inform whether patients with early stage, adverse risk disease defined by a range of factors, benefit from early intervention. However, direct targeting of the malignant cells at an earlier stage may drive mechanisms of resistance, beyond which alternative treatment options are limited.

In view of the fact that CLL cells appear to be ‘addicted’ to the microenvironmental signals in order to proliferate, characterisation of the process of trafficking between the lymph-node and peripheral blood is a key area of investigation. Furthermore, CLL is a disease characterised by significant clinical heterogeneity that reflects key differences in disease biology. We therefore performed a detailed *in-vivo* investigation into the relationship between CLL cell phenotype, function, trafficking and proliferation in order to better define the relationship between these characteristics. This investigation was performed as part of the observational ‘*In-Vivo* Kinetics in Chronic Lymphocytic Leukaemia Study’ that involved *in-vivo* labelling of proliferating cells using deuterated glucose.

The observational study was aligned to the CyCLLe study, we therefore initially included patients with non-progressive CD38+ CLL. However, due to slow recruitment, we amended the protocol to include all patients with non-progressive CLL regardless of CD38 expression. By chance, all patients recruited had mutated *IgV_H* genes, associated with a state of non-responsiveness to agonistic BCR ligation and a favourable overall prognosis (Damle et al., 1999; Hamblin et al., 1999; Mockridge et al., 2007).

The mean proliferation rate in this cohort of patients with non-progressive disease as expected, was slightly lower than that reported by Messmer et al using deuterated water labelling and Defoiche et al using deuterated glucose labelling (Defoiche et al., 2008; Messmer et al., 2005).

We found that proliferation was greatest in patients that expressed CD38 and was unrelated to BCR signalling capacity; two of three patients expressing CD38 had high proliferation rates and neither patient exhibited active signalling through the BCR. The two patients with the highest proliferation rates harboured trisomy 12; importantly the presence of trisomy 12 is associated with expression of CD11c, CD38 and CD49d and a propensity towards migration into the tumour microenvironment (Deaglio et al., 2007; Pasikowska et al., 2016; Riches et al., 2014; Vaisitti et al., 2010). Full phenotypic assessment of the leukaemic cells, including CD49d and CXCR4 expression would have been useful in establishing the relationship between trisomy 12, trafficking and proliferation in these cases.

In our small cohort of patients, we found no direct relationship between sIgM expression, BCR signalling and CLL proliferation rates. A larger cohort study including more patients with active signalling through the BCR would be required to formally investigate this relationship.

Telomere length, previously correlated with risk factors including CD38 expression, *IgV_H* mutational status and advanced clinical stage, was measured to assess the relationship between point proliferation rate and replicative history. Single telomere length analysis (STELA) in our small cohort of patients revealed no clear association between point proliferation and telomere length, although we did note that the patient with the lowest CLL proliferation rate had the longest telomeres. This is at odds with large clinical studies that have reported an association between biological characteristics associated with proliferation and should be investigated in a larger cohort study including patients with UM-CLL (Lin et al., 2010; Rossi et al., 2009).

6.8 *In-vivo* Study of Proliferation and Trafficking of Tumour Cells in Patients with CLL

In view of the fact that proliferation takes place in the lymph-node compartment and that migration into the lymph-node appears to be an important determinant of proliferation, we went on to investigate the trafficking of proliferating cells *in-vivo* using deuterated glucose labelling. ‘Pulse-labelling’ is a unique selling point of deuterated glucose; a ten-hour labelling period achieves a well-defined pulse of labelled cells that can be tracked over time. We measured the appearance of labelled cells in the peripheral blood (release rate) followed by the disappearance of labelled cells by measuring the deuterium enrichment in purified peripheral blood CLL cells. Release rates are of particular interest in CLL given the fact that dysregulation of S1P1 is thought to impair egress of CLL cells (Capitani et al., 2012; Sic et al., 2014) and in view of the profound lymphocytosis observed upon the commencement of BCR antagonists (Herman, Niemann, et al., 2014b).

The release rate of CLL cells was found to be slower than that reported for healthy lymphocytes and slowest in patients with splenomegaly, consistent with studies performed using tritiated thymidine labelling (Zimmerman et al., 1968). Delayed release has previously been associated with higher CXCR4 expression, which, in turn is

associated with inferior clinical outcomes in CLL (Calissano et al., 2009; Pepper et al., 2015). Further investigation into the biology of 'egress' should include measurement of S1P1 expression by deuterium labelled CLL cells.

Disappearance rates in this study were significantly slower than those reported for healthy B lymphocytes, suggesting that impaired apoptosis may play a role in the pathogenesis of CLL with mutated *IgV_H* genes. Crucially, there appeared to be heterogeneous rates of loss of CLL cells within patients, suggestive of sub-clones with discordant proliferative characteristics.

We subsequently looked at the trafficking of recently proliferated cells between the lymph-node and peripheral blood by performing paired sampling at regular intervals in three patients with palpable lymph-nodes. Lymph-node re-entry was observed in one patient in this limited study, a remarkable finding in that it is the first time that lymph-node re-entry has been observed. To investigate this further, more patients should be studied with paired sampling over a longer time-period.

The relationship between the proliferation and trafficking of CLL cells between the lymph-node and peripheral blood compartments is best studied *in-vivo*, where the malignant cells are able to interact with the microenvironment in its entirety. Using *in-vivo* labelling with deuterated water, Calissano et al elegantly demonstrated that recently proliferated CLL cells are characterised by high expression of CD5 and low expression of CXCR4, a chemokine receptor that is downregulated following binding with SDF-1 in the lymph-node (Calissano & Damle, 2011). The authors proposed a model whereby recently proliferated CLL cells re-express CXCR4 and downregulate CD5 expression in the circulation, leading to the re-entry of cells to the lymph-node.

We performed 'pulse-labelling' using deuterated glucose to assess dynamic changes in labelling in peripheral blood sub-clones described by Calissano et al. Remarkably, we identified a non-proliferating sub-clone in all three patients assessed, characterised by CXCR4^{hi}/ CD5 low. There appeared to be continuity between CXCR4 low/ CD5 high and CXCR4^{int}/ CD5^{int} populations, suggestive of re-expression of CXCR4 to an intermediate level in the peripheral blood. However, the sub-population characterised

by CXCR4hi/ CD5low remained unlabelled throughout and did not interact with the other sub-populations suggesting that it is a distinct, quiescent sub-population.

Strikingly, we found evidence of all three sub-populations defined by CXCR4 and CD5 expression in the lymph-node. The population characterised by CXCR4low/ CD5hi was the most highly labelled population and in view of the fact that this closely matched the labelling in the entire cohort of CLL cells, this population accounted for the bulk of CLL cells in the lymph-node. After 28 days, label was lost from the CXCR4low/ CD5hi population and gained in the intermediate population possibly suggesting that CXCR4low/ CD5hi labelled cells had left the lymph-node whilst cells with an intermediate phenotype had entered. Similar to the CXCR4hi/ CD5 low population in the peripheral blood, this population remained unlabelled i.e. did not proliferate in the lymph-node.

In view of the relationship between BCR signalling and proliferation in the lymph-node (Herishanu et al., 2011), we used deuterated glucose labelling to assess proliferation and trafficking in CLL subpopulations defined by sIgM expression. Again, we found that there was minimal labelling in the decade of cells with the lowest sIgM expression, consistent with a state of quiescence. The decade of cells with the highest sIgM expression was highly enriched with labelled cells, with a proliferation rate 30x that of the opposite phenotype. The observation that the sub-population with highest sIgM expression is most enriched with proliferating cells is consistent with reports that lymph-node derived CLL cells are characterised by high expression of sIgM (Coulter, Manuscript submitted) but challenges the hypothesis that sIgM expression is down-regulated upon proliferation (Coelho et al., 2013). Mechanistically, IL4 has recently been reported to induce the expression of sIgM by CLL cells in the lymph-node (Aguilar-Hernandez et al., 2016).

To investigate a functional relationship between sIgM expression and proliferation, we went on to measure the deuterium enrichment in CLL cells according to ability to internalise the BCR. We found that sub-populations of CLL cells sorted according to BCR internalisation mirrored those sorted according to sIgM expression. This observation suggests a process whereby within the lymph-node, CLL cells exhibit

enhanced sIgM expression, BCR internalisation and proliferation. Upon release into the peripheral blood, recently proliferated CLL cells down-regulate sIgM expression and no longer proliferate.

Critically, a sub-population of quiescent cells that appeared to be independent of other sub-populations was identified in all patients. This apparently non-proliferative population had the characteristics of CXCR4^{hi}/ CD5^{low}, sIgM low and low BCR internalisation. In order to investigate whether the proliferative history of the quiescent subpopulation, telomere length was measured in three patients, taking the highest and lowest decade of cells according to BCR internalisation. In the most proliferative patient, with a proliferation rate more than twice that of the other patients, we found that telomere length was 2.2kB longer in the decade with the lowest BCR internalisation. Although conclusions cannot be drawn from a single observation, this may suggest that there is reduced telomere attrition in the quiescent sub-population and should be further investigated.

The non-proliferative population of cells is of particular interest in the era of drugs that inhibit BCR signalling, where it has been observed that up to a third of patients (particularly those with mutated *IgV_H* genes) develop a persistent lymphocytosis (Woyach, Smucker, et al., 2014c). Detailed characterisation of the persistent CLL cells has demonstrated features of anergy, specifically inability to signal through the BCR, constitutive activation of ERK and absent proliferation (Woyach, Smucker, et al., 2014c).

To understand the relationship between the quiescent sub-population observed in our deuterated glucose studies and the persistent population of CLL cells seen in patients treated with ibrutinib, deuterated glucose studies should be performed in patients commencing treatment with this drug. We are yet to learn the significance of the prolonged lymphocytosis in patients receiving ibrutinib therapy; it is currently unclear whether it is an inert sub-population or whether it can undergo genetic evolution and give rise to resistance. One strategy could be to reverse anergy and induce apoptosis through targeted inhibition of NFAT signaling and it would be reasonable to test CsA in this setting.

A larger cohort of patients with mutated *IgV_H* genes should be studied to characterise the phenotype and function of the quiescent sub-population with an emphasis on assessing BCR signalling capacity. Ultimately, targeted sequencing should be performed to assess whether the quiescent sub-population has a distinct genetic profile that underpins its functional characteristics.

Finally, it is necessary to investigate *in-vivo* tumour kinetics in patients with unmutated *IgV_H* genes. Patients with UM-CLL have a shorter time to first treatment and reduced overall survival (Damle et al., 1999; Hamblin et al., 1999). The adverse prognosis is a consequence of distinct biological characteristics including increased signalling through the BCR and proliferation, shorter telomeres and adverse-risk mutations (Herishanu et al., 2011; Hultdin et al., 2003; Mockridge et al., 2007; Rossi et al., 2013). However, it is interesting that patients with UM-CLL respond particularly well to drugs that inhibit signalling through the BCR and do not tend to develop a persistent lymphocytosis (Woyach, Smucker, et al., 2014c). This may be due to the fact that the entire CLL clone undergoes proliferation and that sub-population of quiescent cells does not exist in these patients.

The *in-vivo* studies of tumour kinetics significantly advance the understanding of the relationship between sIgM expression, BCR internalisation, proliferation and subsequent trafficking of CLL cells between the lymph-node and peripheral blood compartments. The 'pulse-labelling' feature of deuterated glucose enabled release and disappearance rates to be studied for the first time in a cohort of well-characterised patients with M-CLL. Although deuterated water studies have demonstrated the importance of proliferation in the pathogenesis of CLL, the deuterated glucose studies presented here provide evidence of long-lived CLL cells and in patients with mutated *IgV_H* genes. This finding revives the debate regarding the role of defective apoptosis versus proliferation in the aetiology of CLL.

There was remarkable heterogeneity in our cohort of ten patients and a larger cohort study is now warranted to further investigate these preliminary findings.

It is hoped that this investigation demonstrates the importance of studying the proliferation and trafficking of tumour cells *in-vivo* in order to accurately characterise the pathogenesis of CLL. By further developing *in-vivo* labelling studies, many more important questions regarding the biology of CLL and impact of targeted therapies could be addressed.

References

- Agathangelidis, A., Darzentas, N., Hadzidimitriou, A., Brochet, X., Murray, F., Yan, X.-J., et al. (2012). Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. *Blood*, 119(19), 4467–4475.
- Aguilar-Hernandez, M. M., Blunt, M. D., Dobson, R., Yeomans, A., Thirdborough, S., Larrayoz, M., et al. (2016). IL-4 enhances expression and function of surface IgM in CLL cells. *Blood*, 127(24), 3015–3025.
- Ahearne, M. J., Willimott, S., Piñon, L., Kennedy, D. B., Miall, F., Dyer, M. J. S., & Wagner, S. D. (2013). Enhancement of CD154/IL4 proliferation by the T follicular helper (Tfh) cytokine, IL21 and increased numbers of circulating cells resembling Tfh cells in chronic lymphocytic leukaemia. *British Journal of Haematology*, 162(3), 360–370.
- Apollonio, B., Scielzo, C., Bertilaccio, M. T. S., Hacken, ten, E., Scarfo, L., Ranghetti, P., et al. (2013). Targeting B-cell anergy in chronic lymphocytic leukemia. *Blood*, 121(19), 3879–3888.
- Bagnara, D., Kaufman, M. S., Calissano, C., Marsilio, S., Patten, P. E. M., Simone, R., et al. (2011). A novel adoptive transfer model of chronic lymphocytic leukemia suggests a key role for T lymphocytes in the disease. *Blood*, 117(20), 5463–5472.
- Baliakas, P., Hadzidimitriou, A., Sutton, L.-A., Rossi, D., Minga, E., Villamor, N., et al. (2015). Recurrent mutations refine prognosis in chronic lymphocytic leukemia. *Leukemia*, 29(2), 329–336.
- Bhattacharya, N., Reichenzeller, M., Caudron-Herger, M., Haebe, S., Brady, N., Diener, S., et al. (2015). Loss of cooperativity of secreted CD40L and increased dose-response to IL4 on CLL cell viability correlates with enhanced activation of NF-κB and STAT6. *International Journal of Cancer*, 136(1), 65–73.
- Binet, J., Auquier, A., Dighiero, G., & Chastang, C. (1981). A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis - Binet - 2006 - Cancer - Wiley Online Library. *Cancer*.
- Borel, J. F., Feurer, C., Gubler, H. U., & Stähelin, H. (1976). Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents and Actions*, 6(4), 468–475.
- Brusa, D., Serra, S., Coscia, M., Rossi, D., D'Arena, G., Laurenti, L., et al. (2013). The PD-1/PD-L1 axis contributes to T-cell dysfunction in chronic lymphocytic leukemia. *Haematologica*, 98(6), 953–963.
- Buggins, A. G. S., Patten, P. E. M., Richards, J., Thomas, N. S. B., Mufti, G. J., & Devereux, S. (2008). Tumor-derived IL-6 may contribute to the immunological defect in CLL. *Leukemia*, 22(5), 1084–1087.
- Buggins, A. G. S., Pepper, C., Patten, P. E. M., Hewamana, S., Gohil, S., Moorhead, J., et al. (2010). Interaction with vascular endothelium enhances survival in primary chronic lymphocytic leukemia cells via NF-κB activation and de novo gene transcription. *Cancer Research*, 70(19), 7523–7533.
- Bulian, P., Shanafelt, T. D., Fegan, C., Zucchetto, A., Cro, L., Nuckel, H., et al. (2014). CD49d Is the Strongest Flow Cytometry-Based Predictor of Overall Survival in Chronic Lymphocytic Leukemia. *Journal of Clinical Oncology* 32:897-904

- Burger, J. A., Burger, M., & Kipps, T. J. (1999). Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. *Blood*, 94(11), 3658–3667.
- Burger, J. A., Tsukada, N., Burger, M., Zvaifler, N. J., Dell'Aquila, M., & Kipps, T. J. (2000). Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood*, 96(8), 2655–2663.
- Bürgler, S., Gimeno, A., Parente-Ribes, A., Wang, D., Os, A., Devereux, S., et al. (2015). Chronic lymphocytic leukemia cells express CD38 in response to Th1 cell-derived IFN- γ by a T-bet-dependent mechanism. *The Journal of Immunology*, 194(2), 827–835.
- Byrd, J. C., Brown, J. R., O'Brien, S., Barrientos, J. C., Kay, N. E., Reddy, N. M., et al. (2014a). Ibrutinib versus ofatumumab in previously treated chronic lymphoid leukemia. *The New England Journal of Medicine*, 371(3), 213–223.
- Byrd, J. C., Jones, J. J., Woyach, J. A., Johnson, A. J., & Flynn, J. M. (2014b). Entering the Era of Targeted Therapy for Chronic Lymphocytic Leukemia: Impact on the Practicing Clinician. *Journal of Clinical Oncology*.
- Calissano, C., & Damle, R. (2011). *Molecular Medicine*, 17(11-12), 1.
- Calissano, C., Damle, R. N., Hayes, G., Murphy, E. J., Hellerstein, M. K., Moreno, C., et al. (2009). In vivo intraclonal and interclonal kinetic heterogeneity in B-cell chronic lymphocytic leukemia. *Blood*, 114(23), 4832–4842.
- Calne, R. Y. (1979). Immunosuppression for organ grafting -- observations on cyclosporin A. *Immunological Reviews*, 46, 113–124.
- Capitani, N., Patrussi, L., Trentin, L., Lucherini, O. M., Cannizzaro, E., Migliaccio, E., et al. (2012). S1P1 expression is controlled by the pro-oxidant activity of p66Shc and is impaired in B-CLL patients with unfavorable prognosis. *Blood*. 120 (22) 4391-4399.
- Chikkappa, G., Pasquale, D., Phillips, P. G., Mangan, K. F., & Tsan, M. F. (1987). Cyclosporin-A for the treatment of pure red cell aplasia in a patient with chronic lymphocytic leukemia. *American Journal of Hematology*, 26(2), 179–189.
- Choi, M. S., Brines, R. D., Holman, M. J., & Klaus, G. G. (1994). Induction of NF-AT in normal B lymphocytes by anti-immunoglobulin or CD40 ligand in conjunction with IL-4. *Immunity*, 1(3), 179–187.
- Chu, C. C., Catterall, R., Hatzi, K., Yan, X.-J., Zhang, L., Wang, X. B., et al. (2008). Chronic lymphocytic leukemia antibodies with a common stereotypic rearrangement recognize nonmuscle myosin heavy chain IIA. *Blood*, 112(13), 5122–5129.
- Coelho, V., Krysov, S., Steele, A., Sanchez Hidalgo, M., Johnson, P. W., Chana, P. S., et al. (2013). Identification in CLL of circulating intraclonal subgroups with varying B-cell receptor expression and function. *Blood*, 122(15), 2664–2672.
- Cortes, J., O'Brien, S., Loscertales, J., Kantarjian, H., Giles, F., Thomas, D., et al. (2001). Cyclosporin A for the treatment of cytopenia associated with chronic lymphocytic leukemia. *Cancer*, 92(8), 2016–2022.
- Cuní, S., Pérez-Aciego, P., Pérez-Chacón, G., Vargas, J. A., Sánchez, A., Martín-Saavedra, F. M., et al. (2004). A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. *Leukemia*, 18(8), 1391–1400.

- D'Arena, G., Musto, P., Cascavilla, N., Dell'Olio, M., Di Renzo, N., Perla, G., et al. (2001). CD38 expression correlates with adverse biological features and predicts poor clinical outcome in B-cell chronic lymphocytic leukemia. *Leukemia & Lymphoma*, 42(1-2), 109–114.
- Damle, R. N., Temburni, S., Calissano, C., Yancopoulos, S., Banapour, T., Sison, C., et al. (2007). CD38 expression labels an activated subset within chronic lymphocytic leukemia clones enriched in proliferating B cells. *Blood*, 110(9), 3352–3359.
- Damle, R. N., Wasil, T., Fais, F., Ghiotto, F., Valetto, A., Allen, S. L., et al. (1999). Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood*, 94(6), 1840–1847.
- Deaglio, S., Capobianco, A., Bergui, L., Dürig, J., Morabito, F., Dührsen, U., & Malavasi, F. (2003). CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. *Blood*, 102(6), 2146–2155.
- Deaglio, S., Vaisitti, T., Aydin, S., Bergui, L., D'Arena, G., Bonello, L., et al. (2007). CD38 and ZAP-70 are functionally linked and mark CLL cells with high migratory potential. *Blood*, 110(12), 4012–4021.
- Defoiche, J., Debacq, C., Asquith, B., Zhang, Y., Burny, A., Bron, D., et al. (2008). Reduction of B cell turnover in chronic lymphocytic leukaemia. *British Journal of Haematology*, 143(2), 240–247.
- Delgado, J., Pratt, G., Phillips, N., Briones, J., Fegan, C., Nomdedeu, J., et al. (2009). Beta 2-microglobulin is a better predictor of treatment-free survival in patients with chronic lymphocytic leukaemia if adjusted according to glomerular filtration rate. *British Journal of Haematology*, 145(6), 801–805.
- Dewald, G. W., Brockman, S. R., Paternoster, S. F., Bone, N. D., O'Fallon, J. R., Allmer, C., et al. (2003). Chromosome anomalies detected by interphase fluorescence in situ hybridization: correlation with significant biological features of B-cell chronic lymphocytic leukaemia. *British Journal of Haematology*, 121(2), 287–295.
- Dighiero, G., Maloum, K., Desablens, B., Cazin, B., Navarro, M., Leblay, R., et al. (1998). Chlorambucil in indolent chronic lymphocytic leukemia. French Cooperative Group on Chronic Lymphocytic Leukemia. *The New England Journal of Medicine*, 338(21), 1506–1514.
- Dohner, H., Fischer, K., Bentz, M., Hansen, K., Benner, A., Cabot, G., et al. (1995). p53 gene deletion predicts for poor survival and non-response to therapy with purine analogs in chronic B-cell leukemias. *Blood*, 85(6), 1580–1589.
- Dohner, H., Stilgenbauer, S., Benner, A., Leupolt, E., Kröber, A., Bullinger, L., et al. (2000). Genomic aberrations and survival in chronic lymphocytic leukemia. *The New England Journal of Medicine*, 343(26), 1910–1916.
- Dohner, H., Stilgenbauer, S., James, M. R., Benner, A., Weilguni, T., Bentz, M., et al. (1997). 11q deletions identify a new subset of B-cell chronic lymphocytic leukemia characterized by extensive nodal involvement and inferior prognosis. *Blood*, 89(7), 2516–2522.
- Eichhorst, B., Fink, A.-M., Bahlo, J., Busch, R., Kovacs, G., Maurer, C., et al. (2016). First-line chemoimmunotherapy with bendamustine and rituximab versus fludarabine, cyclophosphamide, and rituximab in patients with advanced chronic lymphocytic leukaemia (CLL10): an international, open-label, randomised, phase 3, non-inferiority trial. *The Lancet Oncology*, 17(7), 928–942.

- Endo, T., Nishio, M.,ENZLER, T., Cottam, H. B., Fukuda, T., James, D. F., et al. (2007). BAFF and APRIL support chronic lymphocytic leukemia B-cell survival through activation of the canonical NF-kappaB pathway. *Blood*, 109(2), 703–710.
- Fabbri, G., Rasi, S., Rossi, D., Trifonov, V., Khiabani, H., Ma, J., et al. (2011). Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. *The Journal of Experimental Medicine*, 208(7), 1389–1401.
- Farace, F., Orlanducci, F., Dietrich, P. Y., Gaudin, C., Angevin, E., Courtier, M. H., et al. (1994). T cell repertoire in patients with B chronic lymphocytic leukemia. Evidence for multiple in vivo T cell clonal expansions. *Journal of Immunology (Baltimore, Md. : 1950)*, 153(9), 4281–4290.
- Fischer, K., Bahlo, J., Fink, A.-M., Goede, V., Herling, C. D., Cramer, P., et al. (2016). Long-term remissions after FCR chemoimmunotherapy in previously untreated patients with CLL: updated results of the CLL8 trial. *Blood*, 127(2), 208–215.
- Flanagan, W. M., Corthésy, B., Bram, R. J., & Crabtree, G. R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature*, 352(6338), 803–807.
- Fuleihan, R., Ramesh, N., Horner, A., Ahern, D., Belshaw, P. J., Alberg, D. G., et al. (1994). Cyclosporin A inhibits CD40 ligand expression in T lymphocytes. *Journal of Clinical Investigation*, 93(3), 1315–1320.
- Furman, R. R., Asgari, Z., Mascarenhas, J. O., Liou, H. C., & Schattner, E. J. (2000). Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. *Journal of Immunology (Baltimore, Md. : 1950)*, 164(4), 2200–2206.
- Furman, R. R., Sharman, J. P., Coutre, S. E., Cheson, B. D., Pagel, J. M., Hillmen, P., et al. (2014). Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. *The New England Journal of Medicine*, 370(11), 997–1007.
- Garg, R., Wierda, W., Ferrajoli, A., Abruzzo, L., Pierce, S., Lerner, S., et al. (2012). The prognostic difference of monoallelic versus biallelic deletion of 13q in chronic lymphocytic leukemia. *Cancer*, 118(14), 3531–3537.
- Ghia, P., Circosta, P., Scielzo, C., Vallario, A., Camporeale, A., Granziero, L., & Caligaris-Cappio, F. (2005). Differential effects on CLL cell survival exerted by different microenvironmental elements. *Current Topics in Microbiology and Immunology*, 294, 135–145.
- Ghia, P., Strola, G., Granziero, L., Geuna, M., Guida, G., Sallusto, F., et al. (2002). Chronic lymphocytic leukemia B cells are endowed with the capacity to attract CD4+, CD40L+ T cells by producing CCL22. *European Journal of Immunology*, 32(5), 1403–1413.
- Gitelson, E., Hammond, C., Mena, J., Lorenzo, M., Buckstein, R., Berinstein, N. L., et al. (2003). Chronic lymphocytic leukemia-reactive T cells during disease progression and after autologous tumor cell vaccines. *Clinical Cancer Research : an Official Journal of the American Association for Cancer Research*, 9(5), 1656–1665.
- Goede, V., Fischer, K., Busch, R., Engelke, A., Eichhorst, B., Wendtner, C. M., et al. (2014). Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. *The New England Journal of Medicine*, 370(12), 1101–1110.
- Goede, V., Fischer, K., Engelke, A., Schlag, R., Lepretre, S., Montero, L. F. C., et al. (2015). Obinutuzumab as frontline treatment of chronic lymphocytic leukemia: updated results of the CLL11 study. *Leukemia*, 29(7), 1602–1604.

- Goodnow, C. C. (1997). Balancing immunity, autoimmunity, and self-tolerance. *Annals of the New York Academy of Sciences*, 815, 55–66.
- Görgün, G., Holderried, T. A. W., Zahrieh, D., Neuberg, D., & Gribben, J. G. (2005). Chronic lymphocytic leukemia cells induce changes in gene expression of CD4 and CD8 T cells. *Journal of Clinical Investigation*, 115(7), 1797–1805.
- Granziero, L., Ghia, P., Circosta, P., Gottardi, D., Strola, G., Geuna, M., et al. (2001). Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia. *Blood*, 97(9), 2777–2783.
- Guarini, A., Chiaretti, S., Tavolaro, S., Maggio, R., Peragine, N., Citarella, F., et al. (2008). BCR ligation induced by IgM stimulation results in gene expression and functional changes only in IgV H unmutated chronic lymphocytic leukemia (CLL) cells. *Blood*, 112(3), 782–792.
- Guarini, A., Marinelli, M., Tavolaro, S., Bellacchio, E., Magliozzi, M., Chiaretti, S., et al. (2012). ATM gene alterations in chronic lymphocytic leukemia patients induce a distinct gene expression profile and predict disease progression. *Haematologica*, 97(1), 47–55.
- Guo, B., Zhang, L., Chiorazzi, N., & Rothstein, T. L. (2016). IL-4 rescues surface IgM expression in chronic lymphocytic leukemia. *Blood*, 128(4), 553–562.
- Hallek, M. (2013). Chronic lymphocytic leukemia: 2013 update on diagnosis, risk stratification and treatment. *American Journal of Hematology*, 88(9), 803–816.
- Hallek, M., Cheson, B. D., Catovsky, D., Caligaris-Cappio, F., Dighiero, G., Dohner, H., et al. (2008). Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*, 111(12), 5446–5456.
- Hallek, M., Fischer, K., Fingerle-Rowson, G., Fink, A., Busch, R., Mayer, J., et al. (2010). Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *The Lancet*, 376(9747), 1164–1174.
- Hallek, M., Wanders, L., Ostwald, M., Busch, R., Senekowitsch, R., Stern, S., et al. (1996). Serum beta(2)-microglobulin and serum thymidine kinase are independent predictors of progression-free survival in chronic lymphocytic leukemia and immunocytoma. *Leukemia & Lymphoma*, 22(5-6), 439–447.
- Hamblin, T. J., Davis, Z., Gardiner, A., Oscier, D. G., & Stevenson, F. K. (1999). Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood*, 94(6), 1848–1854.
- Hamblin, T. J., Orchard, J. A., Ibbotson, R. E., Davis, Z., Thomas, P. W., Stevenson, F. K., & Oscier, D. G. (2002). CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. *Blood*, 99(3), 1023–1029.
- Hamilton, E., Pearce, L., Morgan, L., Robinson, S., Ware, V., Brennan, P., et al. (2012). Mimicking the tumour microenvironment: three different co-culture systems induce a similar phenotype but distinct proliferative signals in primary chronic lymphocytic leukaemia cells. *British Journal of Haematology*, 158(5), 589–599.

- Hanada, M., Delia, D., Aiello, A., Stadtmauer, E., & Reed, J. C. (1993). bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. *Blood*, 82(6), 1820–1828.
- Herishanu, Y., Perez-Galan, P., Liu, D., Biancotto, A., Pittaluga, S., Vire, B., et al. (2011). The lymph-node microenvironment promotes B-cell receptor signaling, NF- B activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood*, 117(2), 563–574.
- Herman, S. E. M., Gordon, A. L., Hertlein, E., Ramanunni, A., Zhang, X., Jaglowski, S., et al. (2011). Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood*, 117(23), 6287–6296.
- Herman, S. E. M., Gordon, A. L., Wagner, A. J., Heerema, N. A., Zhao, W., Flynn, J. M., et al. (2010). Phosphatidylinositol 3-kinase- δ inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. *Blood*, 116(12), 2078–2088.
- Herman, S. E. M., Mustafa, R. Z., Gyamfi, J. A., Pittaluga, S., Chang, S., Chang, B., et al. (2014a). Ibrutinib inhibits BCR and NF- κ B signaling and reduces tumor proliferation in tissue-resident cells of patients with CLL. *Blood*, 123(21), 3286–3295.
- Herman, S. E. M., Niemann, C. U., Farooqui, M., Jones, J., Mustafa, R. Z., Lipsky, A., et al. (2014b). Ibrutinib-induced lymphocytosis in patients with chronic lymphocytic leukemia: correlative analyses from a phase II study. *Leukemia*, 28(11), 2188–2196.
- Hewamana, S., Alghazal, S., Lin, T. T., Clement, M., Jenkins, C., Guzman, M. L., et al. (2008). The NF- B subunit Rel A is associated with in vitro survival and clinical disease progression in chronic lymphocytic leukemia and represents a promising therapeutic target. *Blood*, 111(9), 4681–4689.
- Hewamana, S., Lin, T. T., Rowntree, C., Karunanithi, K., Pratt, G., Hills, R., et al. (2009). Rel A Is an Independent Biomarker of Clinical Outcome in Chronic Lymphocytic Leukemia. *Journal of Clinical Oncology*, 27(5), 763–769.
- Huang, Y. H., Sojka, D. K., & Fowell, D. J. (2012). Cutting Edge: Regulatory T Cells Selectively Attenuate, Not Terminate, T Cell Signaling by Disrupting NF- B Nuclear Accumulation in CD4 T Cells. *The Journal of Immunology*, 188(3), 947–951.
- Hultdin, M., Rosenquist, R., Thunberg, U., Tobin, G., Norrback, K.-F., Johnson, A., et al. (2003). Association between telomere length and V(H) gene mutation status in chronic lymphocytic leukaemia: clinical and biological implications. *British Journal of Cancer*, 88(4), 593–598.
- Huxford, T., Huang, D. B., Malek, S., & Ghosh, G. (1998). The crystal structure of the IkappaBalpha/NF-kappaB complex reveals mechanisms of NF-kappaB inactivation. *Cell*, 95(6), 759–770.
- Jaksic, O., Kardum Skelin, I., & Jaksic, B. (2010). Chronic lymphocytic leukemia: insights from lymph-nodes & bone marrow and clinical perspectives. *Collegium Antropologicum*, 34(1), 309–313.
- Jaksic, O., Paro, M. M. K., Kardum Skelin, I., Kusec, R., Pejisa, V., & Jaksic, B. (2004). CD38 on B-cell chronic lymphocytic leukemia cells has higher expression in lymph-nodes than in peripheral blood or bone marrow. *Blood*, 103(5), 1968–1969.
- Johnston, J. B., Paul, J. T., Neufeld, N. J., Haney, N., Kropp, D. M., Hu, X., et al. (2004). Role of myeloid cell factor-1 (Mcl-1) in chronic lymphocytic leukemia. *Leukemia & Lymphoma*, 45(10), 2017–2027.

- Kim, H.-P., Korn, L. L., Gamero, A. M., & Leonard, W. J. (2005). Calcium-dependent activation of interleukin-21 gene expression in T cells. *The Journal of Biological Chemistry*, 280(26), 25291–25297.
- Kitada, S., Andersen, J., Akar, S., Zapata, J. M., Takayama, S., Krajewski, S., et al. (1998). Expression of apoptosis-regulating proteins in chronic lymphocytic leukemia: correlations with In vitro and In vivo chemoresponses. *Blood*, 91(9), 3379–3389.
- Klaus, G. G. (1988). Cyclosporine-sensitive and cyclosporine-insensitive modes of B cell stimulation. *Transplantation*, 46(2 Suppl), 11S–14S.
- Kröber, A., Seiler, T., Benner, A., Bullinger, L., Brückle, E., Lichter, P., et al. (2002). V(H) mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. *Blood*, 100(4), 1410–1416.
- Kumagai, M., Coustan-Smith, E., Murray, D. J., Silvennoinen, O., Murti, K. G., Evans, W. E., et al. (1995). Ligation of CD38 suppresses human B lymphopoiesis. *The Journal of Experimental Medicine*, 181(3), 1101–1110.
- Lagneaux, L., Delforge, A., Bron, D., De Bruyn, C., & Stryckmans, P. (1998). Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. *Blood*, 91(7), 2387–2396.
- Lampert, I. A., Wotherspoon, A., Van Noorden, S., & Hasserjian, R. P. (1999). High expression of CD23 in the proliferation centers of chronic lymphocytic leukemia in lymph-nodes and spleen. *Human Pathology*, 30(6), 648–654.
- Landau, D. A., Carter, S. L., Stojanov, P., McKenna, A., Stevenson, K., Lawrence, M. S., et al. (2013). Evolution and Impact of Subclonal Mutations in Chronic Lymphocytic Leukemia. *Cell*, 152(4), 714–726.
- Lanemo Myhrinder, A., Hellqvist, E., Sidorova, E., Söderberg, A., Baxendale, H., Dahle, C., et al. (2008). A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. *Blood*, 111(7), 3838–3848.
- Lanham, S., Hamblin, T., Oscier, D., Ibbotson, R., Stevenson, F., & Packham, G. (2003). Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia. *Blood*, 101(3), 1087–1093.
- Le Roy, C., Deglesne, P. A., Chevallier, N., Beitar, T., Eclache, V., Quettier, M., et al. (2012). The degree of BCR and NFAT activation predicts clinical outcomes in chronic lymphocytic leukemia. *Blood*, 120(2), 356–365.
- Lederman, S., Yellin, M. J., Krichevsky, A., Belko, J., Lee, J. J., & Chess, L. (1992). Identification of a novel surface protein on activated CD4+ T cells that induces contact-dependent B cell differentiation (help). *The Journal of Experimental Medicine*, 175(4), 1091–1101.
- Lin, T. S., Ruppert, A. S., Johnson, A. J., Fischer, B., Heerema, N. A., Andritsos, L. A., et al. (2009). Phase II Study of Flavopiridol in Relapsed Chronic Lymphocytic Leukemia Demonstrating High Response Rates in Genetically High-Risk Disease. *Journal of Clinical Oncology*, 27(35), 6012–6018.
- Lin, T. T., Letsolo, B. T., Jones, R. E., Rowson, J., Pratt, G., Hewamana, S., et al. (2010). Telomere dysfunction and fusion during the progression of chronic lymphocytic leukemia: evidence for a telomere crisis. *Blood*, 116(11), 1899–1907.
- Liu, J., Farmer, J. D., Lane, W. S., Friedman, J., Weissman, I., & Schreiber, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell*, 66(4), 807–815.

- Liu, T.-M., Woyach, J. A., Zhong, Y., Lozanski, A., Lozanski, G., Dong, S., et al. (2015). Hypermorphic mutation of phospholipase C, $\gamma 2$ acquired in ibrutinib-resistant CLL confers BTK independency upon B-cell receptor activation. *Blood*, 126(1), 61–68.
- Macallan, D. C., Asquith, B., Zhang, Y., de Lara, C., Ghattas, H., Defoiche, J., & Beverley, P. C. L. (2009). Measurement of proliferation and disappearance of rapid turnover cell populations in human studies using deuterium-labeled glucose. *Nature Protocols*, 4(9), 1313–1327.
- Macallan, D. C., Fullerton, C. A., Neese, R. A., Haddock, K., Park, S. S., & Hellerstein, M. K. (1998). Measurement of cell proliferation by labeling of DNA with stable isotope-labeled glucose: studies in vitro, in animals, and in humans. *Proceedings of the National Academy of Sciences of the United States of America*, 95(2), 708–713.
- Macian, F. (2005). NFAT proteins: key regulators of T-cell development and function. *Nature Reviews. Immunology*, 5(6), 472–484.
- Marienfeld, R., Neumann, M., Chuvpilo, S., Escher, C., Kneitz, B., Avots, A., et al. (1997). Cyclosporin A interferes with the inducible degradation of NF-kappa B inhibitors, but not with the processing of p105/NF-kappa B1 in T cells. *European Journal of Immunology*, 27(7), 1601–1609.
- Messmer, B. T., Messmer, D., Allen, S. L., Kolitz, J. E., Kudalkar, P., Cesar, D., et al. (2005). In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *Journal of Clinical Investigation*, 115(3), 755–764.
- Meyer, S., Kohler, N. G., & Joly, A. (1997). Cyclosporine A is an uncompetitive inhibitor of proteasome activity and prevents NF-kappaB activation. *FEBS Letters*, 413(2), 354–358.
- Minden, M. D.-V., Übelhart, R., Schneider, D., Wossning, T., Bach, M. P., Buchner, M., et al. (2012). Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature*, 489(7415), 309–312.
- Mockridge, C. I., Potter, K. N., Wheatley, I., Neville, L. A., Packham, G., & Stevenson, F. K. (2007). Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. *Blood*, 109(10), 4424–4431.
- Morabito, F., Damle, R. N., Deaglio, S., Keating, M., Ferrarini, M., & Chiorazzi, N. (2006). The CD38 ectoenzyme family: advances in basic science and clinical practice. *Molecular Medicine*, 12(11-12), 342–344.
- Moreau, E. J., Matutes, E., A'Hern, R. P., Morilla, A. M., Morilla, R. M., Owusu-Ankomah, K. A., et al. (1997). Improvement of the chronic lymphocytic leukemia scoring system with the monoclonal antibody SN8 (CD79b). *American Journal of Clinical Pathology*, 108(4), 378–382.
- Moyer, T. P., Post, G. R., Sterioff, S., & Anderson, C. F. (1988). Cyclosporine nephrotoxicity is minimized by adjusting dosage on the basis of drug concentration in blood. *Mayo Clinic Proceedings*, 63(3), 241–247.
- Muzio, M., Apollonio, B., Scielzo, C., Frenquelli, M., Vandoni, I., Boussiotis, V., et al. (2008). Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. *Blood*, 112(1), 188–195.
- Nishiyama, S., Manabe, N., Kubota, Y., Ohnishi, H., Kitanaka, A., Tokuda, M., et al. (2005). Cyclosporin A inhibits the early phase of NF- κ B/RelA activation induced by CD28 costimulatory signaling to reduce the IL-2 expression in human peripheral T cells. *International Immunopharmacology*, 5(4), 699–710.

- Noelle, R. J., Roy, M., Shepherd, D. M., Stamenkovic, I., Ledbetter, J. A., & Aruffo, A. (1992). A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. *Proceedings of the National Academy of Sciences of the United States of America*, 89(14), 6550–6554.
- Nunes, C., Wong, R., Mason, M., Fegan, C., Man, S., & Pepper, C. (2012). Expansion of a CD8(+)PD-1(+) replicative senescence phenotype in early stage CLL patients is associated with inverted CD4:CD8 ratios and disease progression. *Clinical Cancer Research : an Official Journal of the American Association for Cancer Research*, 18(3), 678–687.
- O'Brien, S. M., Lamanna, N., Kipps, T. J., Flinn, I., Zelenetz, A. D., Burger, J. A., et al. (2015). A phase 2 study of idelalisib plus rituximab in treatment-naïve older patients with chronic lymphocytic leukemia. *Blood*, 126(25), 2686–2694.
- Oestreich, K. J., Yoon, H., Ahmed, R., & Boss, J. M. (2008). NFATc1 regulates PD-1 expression upon T cell activation. *The Journal of Immunology*, 181(7), 4832–4839.
- Olsson, J., Wikby, A., Johansson, B., Löfgren, S., Nilsson, B. O., & Ferguson, F. G. (2000). Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study. *Mechanisms of Ageing and Development*, 121(1-3), 187–201.
- Os, A., Bürgler, S., Ribes, A. P., Funderud, A., Wang, D., Thompson, K. M., et al. (2013). Chronic lymphocytic leukemia cells are activated and proliferate in response to specific T helper cells. *Cell Reports*, 4(3), 566–577.
- Owen, Punt, Stranford (2013). Kuby Immunology. 7th Edition.
- Panayiotidis, P., Jones, D., Ganeshaguru, K., Foroni, L., & Hoffbrand, A. V. (1996). Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro. *British Journal of Haematology*, 92(1), 97–103.
- Pascutti, M. F., Jak, M., Tromp, J. M., Derks, I. A. M., Remmerswaal, E. B. M., Thijssen, R., et al. (2013). IL-21 and CD40L signals from autologous T cells can induce antigen-independent proliferation of CLL cells. *Blood*, 122(17), 3010–3019.
- Pasikowska, M., Walsby, E., Apollonio, B., CUTHILL, K., Phillips, E., Coulter, E., et al. (2016). Phenotype and immune function of lymph-node and peripheral blood CLL cells are linked to transendothelial migration. *Blood*, 128(4), 563–573.
- Patten, P. E. M., Buggins, A. G. S., Richards, J., Wotherspoon, A., Salisbury, J., Mufti, G. J., Hamblin, T. J., & Devereux, S. (2008b). CD38 expression in chronic lymphocytic leukemia is regulated by the tumor microenvironment. *Blood*, 111(10), 5173–5181.
- Patten, P., Devereux, S., Buggins, A., Bonyhadi, M., Frohlich, M., & Berenson, R. J. (2005). Effect of CD3/CD28 bead-activated and expanded T cells on leukemic B cells in chronic lymphocytic leukemia. *Journal of Immunology (Baltimore, Md. : 1950)*, 174(11), 6562–3– author reply 6563.
- Pepper, C., Buggins, A. G. S., Jones, C. H., Walsby, E. J., Forconi, F., Pratt, G., et al. (2015). Phenotypic heterogeneity in IGHV-mutated CLL patients has prognostic impact and identifies a subset with increased sensitivity to BTK and PI3Kδ inhibition. *Leukemia*, 29(3), 744–747.

- Pettitt, A. R., Jackson, R., Carruthers, S., Dodd, J., Dodd, S., Oates, M., et al. (2012). Alemtuzumab in Combination With Methylprednisolone Is a Highly Effective Induction Regimen for Patients With Chronic Lymphocytic Leukemia and Deletion of TP53: Final Results of the National Cancer Research Institute CLL206 Trial. *Journal of Clinical Oncology*, 30(14), 1647–1655.
- Pizzi, M., Boi, M., Bertoni, F., & Inghirami, G. (2016). Emerging therapies provide new opportunities to reshape the multifaceted interactions between the immune system and lymphoma cells, 1–11.
- Puente, X. S., Pinyol, M., Quesada, V., Conde, L., Ordóñez, G. R., Villamor, N., et al. (2011). Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature*, 475(7354), 101–105.
- Puiggros, A., Delgado, J., Rodriguez-Vicente, A., Collado, R., Aventin, A., Luño, E., et al. (2013). Biallelic losses of 13q do not confer a poorer outcome in chronic lymphocytic leukaemia: analysis of 627 patients with isolated 13q deletion. *British Journal of Haematology*, 163(1), 47–54.
- Quesada, V., Conde, L., Villamor, N., Ordóñez, G. R., Jares, P., Bassaganyas, L., et al. (2011). Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. *Nature Genetics*, 44(1), 47–52.
- Quiroga, M. P., Balakrishnan, K., Kurtova, A. V., Sivina, M., Keating, M. J., Wierda, W. G., et al. (2009). B-cell antigen receptor signaling enhances chronic lymphocytic leukemia cell migration and survival: specific targeting with a novel spleen tyrosine kinase inhibitor, R406. *Blood*, 114(5), 1029–1037.
- Rai, K. R., Sawitsky, A., Cronkite, E. P., Chanana, A. D., Levy, R. N., & Pasternack, B. S. (1975). Clinical staging of chronic lymphocytic leukemia. *Blood*, 46(2), 219–234.
- Ramsay, A. G., Clear, A. J., Fatah, R., & Gribben, J. G. (2012). Multiple inhibitory ligands induce impaired T-cell immunologic synapse function in chronic lymphocytic leukemia that can be blocked with lenalidomide: establishing a reversible immune evasion mechanism in human cancer. *Blood*, 120(7), 1412–1421.
- Ramsay, A. G., Johnson, A. J., Lee, A. M., Görgün, G., Le Dieu, R., Blum, W., et al. (2008). Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. *Journal of Clinical Investigation*, 118(7), 2427–2437.
- Ranheim, E. A., & Kipps, T. J. (1993). Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *The Journal of Experimental Medicine*, 177(4), 925–935.
- Rawstron, A. C., Bennett, F. L., O'Connor, S. J. M., Kwok, M., Fenton, J. A. L., Plummer, M., et al. (2008). Monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia. *The New England Journal of Medicine*, 359(6), 575–583.
- Ricca, I., Rocci, A., Drandi, D., Francese, R., Compagno, M., Lobetti Bodoni, C., et al. (2007). Telomere length identifies two different prognostic subgroups among VH-unmutated B-cell chronic lymphocytic leukemia patients. *Leukemia*.
- Riches, J. C., Davies, J. K., McClanahan, F., Fatah, R., Iqbal, S., Agrawal, S., et al. (2013). T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. *Blood*, 121(9), 1612–1621.

- Riches, J. C., O'Donovan, C. J., Kingdon, S. J., McClanahan, F., Clear, A. J., Neuberg, D. S., et al. (2014). Trisomy 12 chronic lymphocytic leukemia cells exhibit upregulation of integrin signaling that is modulated by NOTCH1 mutations. *Blood*, 123(26), 4101–4110.
- Roberts, A. W., Davids, M. S., Pagel, J. M., Kahl, B. S., Puvvada, S. D., Gerecitano, J. F., et al. (2016). Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. *The New England Journal of Medicine*, 374(4), 311–322.
- Roos, G., Kröber, A., Grabowski, P., Kienle, D., Bühler, A., Döhner, H., et al. (2008). Short telomeres are associated with genetic complexity, high-risk genomic aberrations, and short survival in chronic lymphocytic leukemia. *Blood*, 111(4), 2246–2252.
- Rossi, D., Bruscaggin, A., Spina, V., Rasi, S., Khiabani, H., Messina, M., et al. (2011). Mutations of the SF3B1 splicing factor in chronic lymphocytic leukemia: association with progression and fludarabine-refractoriness. *Blood*, 118(26), 6904–6908.
- Rossi, D., Fangazio, M., Rasi, S., Vaisitti, T., Monti, S., Cresta, S., et al. (2012). Disruption of BIRC3 associates with fludarabine chemorefractoriness in TP53 wild-type chronic lymphocytic leukemia. *Blood*, 119(12), 2854–2862.
- Rossi, D., Lobetti Bodoni, C., Genuardi, E., Monitillo, L., Drandi, D., Cerri, M., et al. (2009). Telomere length is an independent predictor of survival, treatment requirement and Richter's syndrome transformation in chronic lymphocytic leukemia. *Leukemia*, 23(6), 1062–1072.
- Rossi, D., Spina, V., Bomben, R., Rasi, S., Dal-Bo, M., Bruscaggin, A., et al. (2013). Association between molecular lesions and specific B-cell receptor subsets in chronic lymphocytic leukemia. *Blood*, 121(24), 4902–4905.
- Röth, A., de Beer, D., Nückel, H., Sellmann, L., Dührsen, U., Dürig, J., & Baerlocher, G. M. (2008). Significantly shorter telomeres in T-cells of patients with ZAP-70+/CD38+ chronic lymphocytic leukaemia. *British Journal of Haematology*, 143(3), 383–386.
- Schmid, M., Merk, B., & Porzolt, F. (1994). Cyclosporin A inhibits cytokine-induced proliferation in B-chronic lymphocytic leukemia. *Leukemia & Lymphoma*, 15(3-4), 317–325.
- Schreiber, S. L. (1991). Chemistry and biology of the immunophilins and their immunosuppressive ligands. *Science*, 251(4991), 283–287.
- Schuh, A., Becq, J., Humphray, S., Alexa, A., Burns, A., Clifford, R., et al. (2012). Monitoring chronic lymphocytic leukemia progression by whole genome sequencing reveals heterogeneous clonal evolution patterns. *Blood*, 120(20), 4191–4196.
- Schuh, K., Avots, A., Tony, H. P., Serfling, E., & Kneitz, C. (1996). Nuclear NF-ATp is a hallmark of unstimulated B cells from B-CLL patients. *Leukemia & Lymphoma*, 23(5-6), 583–592.
- Scielzo, C., Apollonio, B., Scarfo, L., Janus, A., Muzio, M., Hacken, ten, E., et al. (2011). The functional in vitro response to CD40 ligation reflects a different clinical outcome in patients with chronic lymphocytic leukemia. *Leukemia*, 25(11), 1760–1767.

- Seifert, M., Sellmann, L., Bloehdorn, J., Wein, F., Stilgenbauer, S., Dürig, J., & Küppers, R. (2012). Cellular origin and pathophysiology of chronic lymphocytic leukemia. *The Journal of Experimental Medicine*, 209(12), 2183–2198.
- Serrano, D., Monteiro, J., Allen, S. L., Kolitz, J., Schulman, P., Lichtman, S. M., et al. (1997). Clonal expansion within the CD4+CD57+ and CD8+CD57+ T cell subsets in chronic lymphocytic leukemia. *Journal of Immunology*, 158(3), 1482–1489.
- Shiow, L. R., Rosen, D. B., Brdicková, N., Xu, Y., An, J., Lanier, L. L., et al. (2006). CD69 acts downstream of interferon- α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature*, 440(7083), 540–544.
- Sic, H., Kraus, H., Madl, J., Flittner, K.-A., Münchow, von, A. L., Pieper, K., et al. (2014). Sphingosine-1-phosphate receptors control B-cell migration through signaling components associated with primary immunodeficiencies, chronic lymphocytic leukemia, and multiple sclerosis. *The Journal of Allergy and Clinical Immunology*, 134(2), 420–428.
- Simms, P. E., & Ellis, T. M. (1996). Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation. *Clinical and Diagnostic Laboratory Immunology*, 3(3), 301–304.
- Sportoletti, P., Baldoni, S., Cavalli, L., Del Papa, B., Bonifacio, E., Ciurnelli, R., et al. (2010). NOTCH1 PEST domain mutation is an adverse prognostic factor in B-CLL. *British Journal of Haematology*, 151(4), 404–406.
- Sreeramkumar, V., Fresno, M., & Cuesta, N. (2011). Prostaglandin E. *Immunology and Cell Biology*, 90(6), 579–586.
- Steininger, C., Widhopf, G. F., Ghia, E. M., Morello, C. S., Vanura, K., Sanders, R., et al. (2012). Recombinant antibodies encoded by IGHV1-69 react with pUL32, a phosphoprotein of cytomegalovirus and B-cell superantigen. *Blood*, 119(10), 2293–2301.
- Stevenson, F. K., Krysov, S., Davies, A. J., Steele, A. J., & Packham, G. (2011). B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*, 118(16), 4313–4320.
- Su'ut, L., O'Connor, S. J., Richards, S. J., Jones, R. A., Roberts, B. E., Davies, F. E., et al. (1998). Trisomy 12 is seen within a specific subtype of B-cell chronic lymphoproliferative disease affecting the peripheral blood/bone marrow and co-segregates with elevated expression of CD11a. *British Journal of Haematology*, 101(1), 165–170.
- Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., & Ben-Neriah, Y. (1994). JNK is involved in signal integration during costimulation of T lymphocytes. *Cell*, 77(5), 727–736.
- Thornton, P. D., Fernandez, C., Giustolisi, G. M., Morilla, R., Atkinson, S., A'Hern, R. P., et al. (2004). CD38 expression as a prognostic indicator in chronic lymphocytic leukaemia. *The Hematology Journal*, 5(2), 145–151.
- Tsimberidou, A.-M., & Keating, M. J. (2005). Richter syndrome. *Cancer*, 103(2), 216–228.
- Tsytsykova, A. V., Tsitsikov, E. N., & Geha, R. S. (1996). The CD40L promoter contains nuclear factor of activated T cells-binding motifs which require AP-1 binding for activation of transcription. *The Journal of Biological Chemistry*, 271(7), 3763–3770.
- Vaisitti, T., Aydin, S., Rossi, D., Cottino, F., Bergui, L., D'Arena, G., et al. (2010). CD38 increases CXCL12-mediated signals and homing of chronic lymphocytic leukemia cells. *Leukemia*, 24(5), 958–969.

- Venkataraman, L., Francis, D. A., Wang, Z., Liu, J., Rothstein, T. L., & Sen, R. (1994). Cyclosporin-A sensitive induction of NF-AT in murine B cells. *Immunity*, 1(3), 189–196.
- Vlad, A., Deglesne, P. A., Letestu, R., Saint-Georges, S., Chevallier, N., Baran-Marszak, F., et al. (2009). Down-regulation of CXCR4 and CD62L in Chronic Lymphocytic Leukemia Cells Is Triggered by B-Cell Receptor Ligation and Associated with Progressive Disease. *Cancer Research*, 69(16), 6387–6395.
- Walsby, E., Buggins, A., Devereux, S., Jones, C., Pratt, G., Brennan, P., et al. (2014). Development and characterization of a physiologically relevant model of lymphocyte migration in chronic lymphocytic leukemia. *Blood*, 123(23), 3607–3617.
- Wang, L., Lawrence, M. S., Wan, Y., Stojanov, P., Sougnez, C., Stevenson, K., et al. (2011). SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. *The New England Journal of Medicine*, 365(26), 2497–2506.
- Wendtner, C. M., Hallek, M., Fraser, G. A. M., Michallet, A.-S., Hillmen, P., Dürig, J., et al. (2016). Safety and efficacy of different lenalidomide starting doses in patients with relapsed or refractory chronic lymphocytic leukemia: results of an international multicenter double-blinded randomized phase II trial. *Leukemia & Lymphoma*, 1–9.
- Wikby, A., Johansson, B., Olsson, J., Löfgren, S., Nilsson, B. O., & Ferguson, F. (2002). Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. *Experimental Gerontology*, 37(2-3), 445–453.
- Willimott, S., Baou, M., Huf, S., Deaglio, S., & Wagner, S. D. (2007). Regulation of CD38 in proliferating chronic lymphocytic leukemia cells stimulated with CD154 and interleukin-4. *Haematologica*, 92(10), 1359–1366.
- Wortis, H. H., Teutsch, M., Higer, M., Zheng, J., & Parker, D. C. (1995). B-cell activation by crosslinking of surface IgM or ligation of CD40 involves alternative signal pathways and results in different B-cell phenotypes. *Proceedings of the National Academy of Sciences of the United States of America*, 92(8), 3348–3352.
- Woyach, J. A., Bojnik, E., Ruppert, A. S., Stefanovski, M. R., Goettl, V. M., Smucker, K. A., et al. (2014a). Bruton's tyrosine kinase (BTK) function is important to the development and expansion of chronic lymphocytic leukemia (CLL). *Blood*, 123(8), 1207–1213.
- Woyach, J. A., Furman, R. R., Liu, T.-M., Ozer, H. G., Zapatka, M., Ruppert, A. S., et al. (2014b). Resistance Mechanisms for the Bruton's Tyrosine Kinase Inhibitor Ibrutinib. *The New England Journal of Medicine*, 370(24), 2286–2294.
- Woyach, J. A., Smucker, K., Smith, L. L., Lozanski, A., Zhong, Y., Ruppert, A. S., et al. (2014c). Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. *Blood*. 123 :1810-1817
- Yao, C., Sakata, D., Esaki, Y., Li, Y., Matsuoka, T., Kuroiwa, K., et al. (2009). Prostaglandin E2–EP4 signaling promotes immune inflammation through TH1 cell differentiation and TH17 cell expansion. *Nature Medicine*, 15(6), 633–640.

- Zenz, T., Kröber, A., Scherer, K., Häbe, S., Bühler, A., Benner, A., et al. (2008). Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. *Blood*, 112(8), 3322–3329.
- Zhou, B., Cron, R. Q., Wu, B., Genin, A., Wang, Z., Liu, S., et al. (2002). Regulation of the murine Nfatc1 gene by NFATc2. *The Journal of Biological Chemistry*, 277(12), 10704–10711.
- Zimmerman, T. S., Godwin, H. A., & Perry, S. (1968). Studies of leukocyte kinetics in chronic lymphocytic leukemia. *Blood*, 31(3), 277–291.
- Zucchetto, A., Caldana, C., Benedetti, D., Tissino, E., Rossi, F. M., Hutterer, E., et al. (2013). CD49d is overexpressed by trisomy 12 chronic lymphocytic leukemia cells: evidence for a methylation-dependent regulation mechanism. *Blood*, 122(19), 3317–3321.
- Zupo, S., Isnardi, L., Megna, M., Massara, R., Malavasi, F., Dono, M., et al. (1996). CD38 expression distinguishes two groups of B-cell chronic lymphocytic leukemias with different responses to anti-IgM antibodies and propensity to apoptosis. *Blood*, 88(4), 1365–1374.